

**BIOLOGY AND GENETIC CHARACTERISATION
OF *Burkholderia gladioli* pv. *agaricicola*, THE
CAUSAL ORGANISM OF ‘CAVITY DISEASE’ OF
WHITE BUTTON MUSHROOMS.**

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of
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University of Canterbury
2004

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Dedicated to the memory of my father,

Dr Somenath Roy Chowdhury,

whose inspiration is continually felt in everything I do.

BOOKS ARE THE FRIENDS OF THE MIND

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List of Abbreviations

°C	degrees Celsius
cav ^{+/-}	cavity ^{+/-}
cm	centimetre
bp	base pairs
DDT	1,1' -(2,2,2-Trichloroethylidene)bis [4-chlorobenzene; 1,1,1-trichloro-2,2bis (p-chlorophenyl) ethane
dH ₂ O	double distilled water
GSP	General Secretory Pathway
hr	hour(s)
IR	infra red
mA	milliamperes
mbar	millibar
mm	millimetre
M	molar concentration
LB	Luria Bertani broth
kb	kilobase
kD	kilodalton
mg	milligram
min	minute(s)
μL	micro liters
nm	nanometer
dNTP	2' -deoxy nucleotide triphosphate
ddNTP	2'-dideoxy nucleotide triphosphate
OD _{xxx}	optical density at xxx nanometers
ORF	open reading frame
Prot ^{+/-}	Protease secretion +/-
PVC	polyvinyl chloride
rpm	revolution per minute
RT	room temperature

SDS	sodium dodecyl sulphate
sdH ₂ O	sterile double distilled water
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
sec	second(s)
UV	ultra violet
vol	volume
v/v	volume per volume
w/v	weight per volume

Abstract

‘Cavity disease’, defined as the rapid degradation of the sporocarps of white button mushrooms, is caused by *Burkholderia gladioli* pv. *agaricola*, a member of Pseudomonaceae. The specific strain analysed in this thesis, BG164, was isolated from New Zealand, but the disease has also been sporadically reported in Europe.

Nine mutants that did not cause cavity symptoms (‘no-cavity’) were isolated by transposon mutagenesis of the BG164 derivative, BG164R. Eight mutants had altered flagella number, did not secrete protease, had a highly reduced capacity to secrete chitinase and did not degrade mushroom mycelia. All these eight mutations were clustered in the GSP (General Secretory Pathway) operon. A cosmid, carrying 23.4kb genomic DNA, pCosGSP, complemented all mutations, which along with mapping of the cosmid, confirms that the cosmid has most or all of the functional *gsp* operon.

The ninth mutant, BGII-107, secreted protease, although it did not form cavity on mushrooms. The DNA sequence analysis suggests that the mutation is in a polyketide synthetase gene. Interestingly, the mutant can also be complemented by the *gsp* operon-containing cosmid, suggesting the presence of a polyketide synthetase gene adjacent to the GSP genes.

Cavity disease initiation, in contrast to most of the other diseases, requires a very high inoculum of the pathogen, suggesting a probable reason for the sporadic appearance of the disease. Studies on the indigenous non-pathogenic bacterial population of mushrooms led to the identification of a natural antagonist of BG164R, *Ewingella americana* (PRC120), in the sporocarp tissue. Population studies indicated the involvement of polymicrobial interactions and a quorum sensitivity-dependent induction of Cavity disease expression.

Chapter I

General Introduction.

1.1. Introduction.

“The distinct difference between commensal, opportunistic and pathogenic microbes is that pathogenic microbes have evolved the genetic ability to breach cellular and anatomic barriers that ordinarily restrict other microorganisms. Thus pathogens can inherently cause damage to cells to forcefully gain access to a new, unique niche that provides them with less competition from other microorganisms, as well as with a ready new source of nutrients”- Stanley Falkow (Falkow, 1998).

Microorganisms were probably the first forms of life on earth. In the process of adapting to the changing environmental conditions over the past 3.8 billion years (Mojzsis *et al.*, 1996; Nutman *et al.*, 1997) they have evolved to be ubiquitous. Microbial populations play an important role in maintaining the ecological balance of nature, while primarily still being engaged in their struggle for existence.

Microbial interactions in nature can be broadly divided into eight classes – neutralism, amensalism, commensalism, synergism, mutualism, competition, predation and parasitism. All these interactions are widely evident in a variety of natural habitats. When living hosts serve as specific niches, and interactions between host and microbe cause damage to the host (Brogden & Guthmiller, 2003), the interactions are called “diseases” and the microbes are known as “pathogens”. However, if we consider it from the point of view of the microbe, it could simply be stated as a strategy adopted for survival in a specific niche.

The term ‘disease’ implies to the ‘manifestation of damage to host’ resulting from host microbe interactions (Casadevall & Pirofski, 2000). The basic principle underlying disease expression is the ‘genetic capacity’ of a ‘pathogen’ to ‘breach host’s defence mechanisms’ (Falkow, 1998). The ability of bacteria to elicit disease symptoms in a host is either an attribute of signals from a potential host or a strategy adopted by the pathogen to survive in the host. Thus, regardless of the initial cause, bacteria become pathogens by responding to stimuli and switching on certain gene(s) that is/are detrimental to the host, but beneficial to the pathogen.

Disease is observed in all forms of living organisms, including bacteria, if we consider phage infections that lyse bacterial cells as viral diseases. Depending on the nature of the host, microorganisms adopt various strategies to optimise their interactions with the hosts in order to cause diseases. These include production of a wide range of products including enzymes, toxins and secondary metabolites that are collectively known as virulence factors (Salmond, 1994; Finlay & Falkow, 1997). A basic theme of disease formation is the release of these virulence factors by the pathogen into the targeted host tissue. Thus, secretion of virulence factors forms an essential component of disease expression, especially in case of bacterial pathogens. This thesis describes an attempt to identify the virulence factors involved in the expression of ‘Cavity disease’ in white button mushrooms.

In 1992, Gill and Cole reported a novel disease in white button mushrooms, *Agaricus bisporus* (Lange) Imbach, from a farm in New Zealand. The disease rapidly affected the sporophores resulting in marked tissue degradation, and in severe cases caused a deep cavity in the cap of mushroom fruit body. Due to the typical symptoms associated, the disease was named ‘Cavity disease’ (Figure 1.1.), and, was defined as the ‘rapid degradation of the pileus tissue of white button mushrooms’. The causative organism was identified as *Pseudomonas cepacia* and the strain was designated CANU-PMS164 (Gill & Cole, 1992).

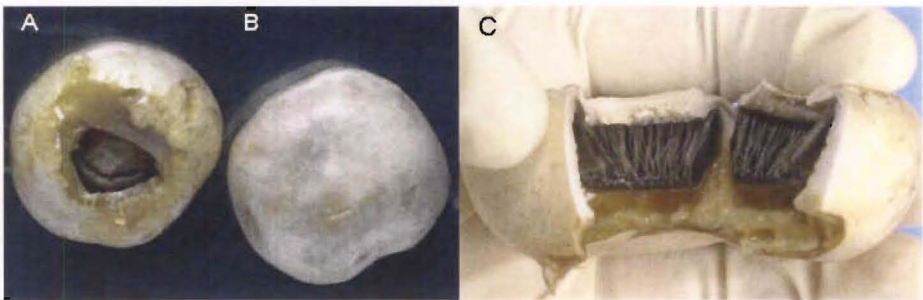


Figure 1.1: Cavity disease of button mushroom, [A] diseased mushroom. [B] healthy mushroom. [C] lateral view of a diseased mushroom cavity, showing unaffected gills.

Unlike most of the predominant mushroom diseases causing significant crop losses worldwide (discussed in section 1.3.4.), this disease is not associated with tissue browning of the mushrooms. The other characteristic feature of this disease is, the pathogen selectively degrades the pileus tissue (Figure 1.2.) of button mushrooms leaving the gills and the stipe intact.

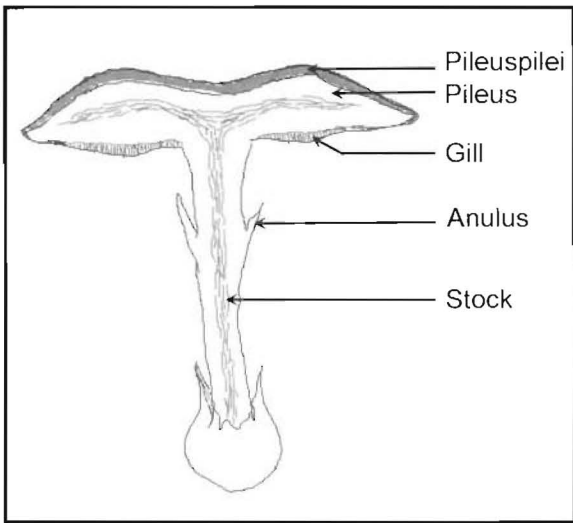


Figure 1.2: Schematic representation of the parts of a mushroom sporocarp

1.2. Why study a mushroom disease?

A. bisporus (Lange) Imbach, the common cultivated button mushroom, is grown worldwide. In Europe, the annual production of *A. bisporus* is $8\text{--}9 \times 10^5$ tonnes per year. The highest producers are France and the Netherlands, followed by the UK (Godfrey, 2003). The New Zealand mushroom industry currently produces 7500 tonnes of button mushrooms per year (Buchanan & Barnes, 2002) for local consumption as well as for export, with an estimated value of five million dollars (New Zealand). Because the worldwide production of cultivated mushrooms continues to grow [(Courvoisier, 1999) cited by Godfrey 2003, (Royse, 1997)], mushroom farmers continuously face the challenge of protecting their crops from diseases and adverse factors that impede the maximum cropping yield. Consequently, the scientific community remains attentive to the problems associated with the industry and interested in suggesting probable remedies.

Problems associated with the yields of *A. bisporus* crops can be attributed to abiotic and biotic factors involved in mushroom cultivation. Abiotic factors for the commercial mushroom production include temperature, relative humidity (Godfrey, 2003) and compost composition (Ivors *et al.*, 2000; Milenkovic *et al.*, 2000) and depend on the specific type of mushroom cultivated. These are constant factors in mushroom cultivation, for which scientifically formulated conditions best suited for the specific type of cultivated mushroom can be adopted in modern mushroom farms (Wong & Preece, 1980). In contrast, the biotic factors associated with decrease in cropping yields are variable and are continuously monitored by producers and scientists (discussed in section 1.3.3. and 1.3.4.). A virus resistant variant of *A. bisporus*, *Agaricus bitorquis* (Quelet) Saccardo, has been introduced in some commercial farms, particularly in tropical countries, and also in some New Zealand farms (Gill & Cole, 1992; Buchanan & Barnes, 2002) to prevent economic losses (Gill & Cole, 1992) due to viral infections. In spite of being virus resistant, *A. bitorquis* is susceptible to a range of bacterial diseases, especially diseases caused by *Pseudomonas* spp (Wong *et al.*, 1982; Rainey & Cole, 1988; Geels *et al.*, 1994; Moquet *et al.*, 1996; Gill & Cole, 2000). Cavity disease

reported by Gill and Cole (1992) was one such pseudomonad disease, and the first example of a non-fluorescent pseudomonad associated with a mushroom disease.

Although Gill and Cole named the disease as 'Cavity disease' in 1992, similar mushroom diseases had been reported earlier from the Horticultural Research International, Littlehampton, U.K. by Lincoln *et al.*, in 1991 (Lincoln *et al.*, 1991). They described the disease as 'Rapid soft rot disease of edible mushroom' and identified the causative organism as *Pseudomonas gladioli* pv. *agaricicola*. One of these isolates, strain RR3 was deposited in the National Collection of Plant Pathogenic Bacteria (accession number NCPPB 3850). The soft rot disease in mushrooms is stated to be less prevalent than most of the other Pseudomonad diseases in the mushroom industry. It is sporadically reported from farms in the UK and other European countries. However, when Cavity disease occurs, it causes devastating effects within very short periods of time. Thus it has received considerable attention from mushroom scientists, as a pathogen that has the potential to cause considerable crop losses to the mushroom industry (Fermor & Lincoln, 2001) (Lincoln *et al.*, 1991; Gill & Cole, 1997).

Intrigued by the rate and magnitude of tissue degradation of mushroom sporocarps by *P. gladioli* pv. *agaricicola* strain CANU-PMS164, the current project was initiated to study the molecular mechanism(s) by which the pathogen expresses Cavity disease symptoms on button mushrooms. A number of speculations on the probable mode of action of CANU-PMS164 in the formation of Cavity disease was suggested by Gill and Cole, (1997) on the basis of biological experiments. It was shown that the strain produces a potent inhibitor of tyrosinase (Ferrar, 1995), which is the enzyme responsible for the browning of tissues in mushrooms and other plants. However, to date, genetic analyses directed towards identifying the actual gene(s) or gene product(s) of *P. gladioli* pv. *agaricicola* or *P. cepacia* CANU-PMS164 responsible for disease expression have not been undertaken. Thus, molecular investigations addressing the gene(s) involved in expression of Cavity disease form the main theme of this thesis.

1.3. Literature review.

1.3.1. Previous research on the causative agent of Cavity disease.

In the first report of Cavity disease by Gill and Cole in 1992, the causative microorganism was designated as CANU-PMS164 and deposited in the culture collection of the Department of Plant and Microbial Sciences, University of Canterbury. In spite of a few differences with the type strain ATCC 25416, CANU-PMS164 was identified as *P. cepacia* and was the first report of a non-fluorescent pseudomonad associated with mushroom disease. The range of symptoms observed in case of cavity disease varied from mild blotching to pitting of the fruiting body of mushroom sporocarp; and, in acute cases led to the complete degradation of the whole sporocarp (Gill & Cole, 1992).

Young (1992) surveyed antifungal compounds produced by soil-inhabiting microbes. CANU-PMS 164 was one of the isolates investigated and showed that this strain inhibits the growth of filamentous fungi, *Trichophyton* and *Fusarium*. On the basis of some biochemical studies, including thin layer chromatography and UV absorption spectra, it was speculated that the inhibitory compound was pyrrolnitrin (a common antifungal antibiotic produced by *Pseudomonas* sp.), or a closely related compound for which biological activity is destroyed at temperatures above 40°C (Young, 1992).

Ferrar (1995) also screened CANU-PMS164 when he was surveying phytopathogens as a source of novel diphenol oxidase (DPO) inhibitors. DPO is responsible for the browning of most fruits and vegetables. Ferrar demonstrated that CANU-PMS164 causes 20% inhibition of the mushroom *ortho*-diphenol oxidase (*o*-DPO) or tyrosinase but does not inhibit the *para*-diphenol oxidase (*p*-DPO) or laccase (Ferrar, 1995).

Gill and Tsuneda (1997) investigated the pathogenicity of CANU-PMS164 on other varieties of economically important mushrooms in Japan such as *Lentinula edodes*, *Pleurotus ostreatus*, *Flammulina velutipes*, *Pholiota nameko*, *Hypsizygus marmoreus*

and *Grifola frondosa*. This study showed that pathogenicity of the organism has a broad host range and is can cause similar soft rotting symptoms in a number of commercially important mushrooms. However, the intensity of disease formation varies amongst the different species. Scanning electron microscopy revealed that damage to the host tissues of the different mushroom species was distinct for each type of mushroom used. The authors suggested that the pathogen damages the various mushroom fruit bodies and their different parts in two ways. Degradation of the hyphal wall layer followed by the degradation of chitin matrix of *A. bisporus* was suggested to be the first mode of action, while shrivelling of mushroom hyphae was the second. The other interesting observation about this pathogen was that it inhibited the mycelial growth in plate assays with mycelial cultures from the different mushroom species. Based on these observations Gill and Tsuneda (1997) speculated that the microorganism first secretes a toxin followed by induction of hyphae degrading enzymes (Gill & Tsuneda, 1997).

In 1997, Gill and Tsuneda reported that the Cavity disease-causing isolate CANU-PMS164 was compared to the mushroom soft rot pathogen *P. gladioli* pv. *agaricicola* (Lincoln *et al.*, 1991) and re-identified as *P. gladioli* pv. *agaricicola* (Gill & Tsuneda, 1997). In 1992, Yabucchi *et al.* (Yabuchi *et al.*, 1992), proposed a new genus name, *Burkholderia*, to include members of the “pseudomallei group”, which were not true Pseudomonads. Based on their proposal, *P. gladioli* pv *agaricicola* CANU-PMS164, was transferred to the new genus and is presently known as *Burkholderia gladioli* pv. *agaricicola*.

At the outset of this project, a 16s rDNA sequence analyses of CANU-PMS164 was carried out to confirm the taxonomic status. On the basis of the analysis and all the information available, the strain was re-designated as BG164. BG stands for *Burkholderia gladioli* pv. *agaricicola*. The collection number, 164, as catalogued earlier, was retained. Subsequently, a spontaneous rifampicin resistant mutant strain of BG164 was generated (section 2.3.1) and this was used in the studies described in this thesis. This strain was identified as BG164R, where R represents Rifampicin resistance in BG164.

1.3.2. Nomenclature of *Burkholderia gladioli* pv. *agaricicola*.

Burkholderia is a new genus name proposed by Yabucchi *et al.* in 1992 for the RNA homology group II Pseudomonads, in honour of the American bacteriologist W. H. Burkholder, who first discovered the etiologic agent of rotten onion (Burkholder, 1950). The genus *Pseudomonas* consists of pathogenic bacteria, mainly plant pathogens and a few animal pathogens. Palleroni *et al.* in 1973 (Palleroni, 1984) divided the different species belonging to the genus *Pseudomonas* into five definitive groups on the basis of rRNA/DNA homology. This system of classification was confirmed subsequently by several laboratories following several other approaches, including investigations on metabolic pathways and their regulatory mechanisms, determination of amino acid sequences of selected proteins, immunological studies, cell wall composition and 16S ribosomal RNA sequences (Palleroni, 1984).

B. gladioli was originally identified as a phytopathogen of gladiolus (Mc Culloch, 1921). The disease symptoms, although often confined to the lower leaves of the plant, could occur in all parts of the foliage. The pathogen was particularly reported to be active under moist and humid conditions, and typical symptoms consisted of circular to elliptical rusty red lesions, which gradually turned dull brown to purple. Lucia Mc Culloch (1921) of the Bureau of Plant Industry in the U.S. Department of Agriculture first isolated the disease causing bacterium of gladioli and named it *Bacterium marginatum*. According to her report, the disease was especially prevalent in the District of Columbia where 80 to 90% of the crops were attacked, although it was found from Illinois to California. Mc Culloch initially isolated and described the phytopathogen, but, it was not chosen as the type strain.

Previously, Severini reported a similar disease in gladiolus and named the causal organism as *P. gladioli* {Severini 1913}. *Bacterium marginatum* (Mc Culloch, 1921) and *P. gladioli* were considered synonyms. Strains with similar phenotypic characteristics have been reported to be pathogenic to iris, onion and gladiolus elsewhere but have mainly been described as *P. marginata*. Burkholder isolated another group of strains, exclusively pathogenic to onions, which was phenotypically very

similar to the strain described above, and named it *P. alliicola* (Palleroni, 1984). Young *et al.* (1978) proposed the inclusion of two pathovars within the species to designate the different pathogenic capacity (Young *et al.*, 1978). The two pathovars of *P. gladioli* suggested were pv. *gladioli* and pv. *alliicola*. The type strain ATCC10248 was chosen as a lectotype to represent *P. gladioli* pv. *gladioli* and the reference strain for *P. gladioli* pv. *alliicola* was ATCC19302 (Palleroni, 1984). The isolation of the mushroom soft rotting bacteria by Lincoln *et al.* in 1991 was the first report of any disease of fungi caused by the rRNA homology group II Pseudomonad. The isolate was compared to the above two pathovars of *P. gladioli* and the creation of a new pathovar, pv. *agaricicola* was proposed within the species *P. gladioli*, to accommodate this newly characterised pathogen. The literal meaning of the term “agaricicola” was “dwelling on the gill bearing fungi” (Lincoln *et al.*, 1991).

1.3.3. Chemical composition of mushroom sporocarps.

Chemically, mushrooms are made up of 89-94% water; 2.6-4% protein, 2.5-5.8% carbohydrates, only 0.2-0.7% crude fat, 0.6-1% fibre and just over 1% ash (Godfrey, 2003). The fungal cell wall can be described as a β -linked chitin and glucan skeleton embedded in an amorphous matrix made up mainly of water-soluble polysaccharides (Alexopoulos *et al.*, 1996). The major carbohydrate is chitin, which is chemically defined as a β -1-4 linked homopolymer of *N*-acetylglucosamine. Chitin is chemically unique to fungal cell walls and insects, and serves a vital role as the main structural component of the fungal cell walls. The glucans in the fungal cell wall are generally long chains of β -1-3 linked glucose molecules with occasional β -1-6 linkages found in the mature cell walls (Carlile *et al.*, 2001). Besides chitin and glucans, other components making up fungal cell walls consist of polysaccharides such as α -glucans and glycoproteins as well as lipids, melanins, D-galactosamine polymers and polyuronids (Alexopoulos *et al.*, 1996). Fungi generally do not have cellulose, a cell wall component prevalent in plants, and pectins, with the exception of members belonging to Oomycetes (Carlile *et al.*, 2001).

Mushrooms are a good source of nutrition for humans, because of their chemical composition (Vetter, 2003). Both the white and brown varieties of *A. bisporus* are

chemically similar, with chitin being the main source of dietary fibre. It has been estimated that chitin provides 27kcal of energy per 100g (Mattila *et al.*, 2002). The major soluble carbohydrates are mannitol, trehalose and glycogen. Sucrose, glucose, and hemicellulose are also present (Burton *et al.*, 1994; Eastwood *et al.*, 2000). Mushroom contains both saturated and unsaturated lipids, about 50% of which are neutral lipids (Godfrey, 2003). They also contain almost all amino acids, and the protein content in mushrooms has been found to be comparably higher than common fruit and vegetables (Zivanovic *et al.*, 2003). Of the minerals, mushrooms contain large amounts of potassium, phosphorous, copper, iron and low concentration of sodium (Vetter, 2003). Nutritionally mushrooms are very healthy and thus are a popular food item for human beings (Zivanovic *et al.*, 2000).

Parameters defining the quality of mushrooms in case of commercial production consist of the colour, appearance, texture, aroma and flavour (Burton *et al.*, 2000), which are, in turn, dependent on the chemical composition of mushrooms. An important aspect governing mushroom quality is the fact that mushroom sporocarps continue to grow and carry on their basic biological processes even after being harvested. It was demonstrated that during the first six days following harvest the mushroom sporocarps toughen in texture, gradually accompanied by softening (Zivanovic *et al.*, 2000). Zivanovic *et al.* (2000), in their work on the textural changes in *A. bisporus*, have ascribed softening of mushroom tissues to the loss of total protein and polysaccharide content and shrinkage of hyphae, leading to an increase in intercellular spaces of pileus tissues, while the toughening was correlated to the increase in the chitin content of mushroom tissues on storage. The preferred colour of mushroom sporocarps is white. However, the most frequent problem associated with the mushroom industry is browning of mushrooms (Mamoun *et al.*, 2000a).

Browning of the mushroom tissue, like most other fruit and vegetables, is a biochemical reaction, which has widely been accepted as a defence mechanism in plants against the pathogens (Walker & Ferrar, 1998). It is an enzymatic process catalysed by two major groups of enzymes, the diphenol oxidases (DPOs) and the peroxidases (Ferrar & Walker, 1999). In intact cells, both the enzyme and its substrates are present, but they are spatially separated, with the enzyme being cytosolic and the phenolic substrates

being present within the vacuoles (Walker & Ferrar, 1998). However, wounds cause a disruption of the vacuolar membrane, thereby bringing the enzyme and substrate into contact, catalysing the reaction and generation of reactive quinones by DPOs (Walker & Ferrar, 1998). These reactive quinones polymerise either with themselves or with amino acids or small peptides to give a characteristic brown colour (Walker, 1975). This protects the tissue: firstly, by sealing off the site of wound, forming a physical barrier and, secondly, by polymerising both the host's proteins and the toxic compounds produced by the pathogen, thereby rendering them inactive (Walker & Ferrar, 1998). DPOs are of two broad types: *ortho*-diphenol oxidases and *para*-diphenol oxidases (Walker, 1975). Although mushrooms have both *o*-DPO and *p*-DPO, their relative distribution in the skin layer (*pileuspilei*) and the soft flesh underneath (*pileus tissue*) are quite different. High levels of *o*-DPO activity are present in the skin of the sporocarp (Burton *et al.*, 1993).

Aldridge and Walker (1980) monitored the changes in the amino acid content during the developmental stages of basidiocarps in an attempt to find out the possible substrate for the activity of *o*-DPO. They found that there was a decrease in the content of tyrosine and phenylalanine and an increase in proline, histidine and threonine with the maturity of the sporocarps. These extracted amino acids were also challenged with the mushroom tyrosinase, but no obvious target could be identified by this approach (Aldridge & Walker, 1980).

Sporophores of mushroom contain both active and latent or inactive tyrosinase (Burton, 1988, Burton *et al.*, 1993). The effects of pre- and post-harvest development of mushroom tyrosinase at different stages as well as on the two different tissue types, namely the skin of the fruiting body (*pileispileis*) and flesh (*pileus trauma*) were investigated by Burton (Burton, 1988). He reported that there were no significant changes in the tyrosinase activity between the different harvests of the mushroom crop, but a major difference lies in the degree of activation of the latent tyrosinase during the pre- and post-harvest stages. Tyrosinase activity increases during the post harvest stage. It was suggested that this change in the tyrosinase activity was due to the increase in the protease activity in the post harvest senescence stage of the mushroom sporocarps. The

tyrosinase activity was found to be about 2 to 2.8 times higher in skin of the mushroom than in the flesh (Burton *et al.*, 1993).

1.3.4. Mushroom diseases.

The diversification of the mushroom industry, in terms of variety of mushrooms as well as production worldwide, has gained impetus since the 1980s (Royse, 1997). With the emergence of this agro-industry, the scientific community has become aware of the different diseases related to mushrooms. Moulds and fungi, viruses and bacteria all cause diseases in mushrooms, resulting in significant economic losses to the mushroom industry.

Fungal diseases:

Fungal diseases, which have been reported in the literature, include ‘Cobweb disease’ caused by *Cladobotryum dendroids* (Mc Kay *et al.*, 1999), ‘Green mould epidemic’ caused by several strains of *Trichoderma* (Mamoun *et al.*, 2000b), ‘Wet bubble disease’ caused by *Mycogone perniciosa* (Umar *et al.*, 2000), ‘Dry bubble disease’ caused by *Verticillium* species (Mills *et al.*, 2000) and ‘Shaggy stipe disease’ caused by *Mortierella bainieri* (Fletcher, 1973).

The ‘Cobweb disease’ is so named after the coarse mycelia covering the affected mushrooms, thereby giving the appearance of a cobweb. This is accompanied by spotting of the caps of the mushroom and is often mistaken for bacterial blotch of the mushrooms. The disease is best identified when conidia from the cobweb rapidly colonise the casing surface and eventually the affected mushrooms turn brown and rot (Mc Kay *et al.*, 1999; Godfrey, 2003).

The ‘Green mould epidemic’ (Mamoun *et al.*, 2000b) is gradually becoming well known as a destructive disease of cultivated mushrooms, causing alarming crop losses in many regions of the world. The disease can easily be recognised by its dark green sporulation in the compost or in the casing layer. Mushrooms normally do not grow in

the affected compost. However, if the mould advances in a mushroom bed, they spot the mushrooms and show disease symptoms similar to 'Mild dew'.

The 'Wet bubble disease' of mushrooms is caused by *Mycogone perniciosa* (Umar *et al.*, 2000). The symptoms are characterised by severe developmental errors in the fruit body formation and acute malformation of the mushroom sporocarps. The disease is very contagious and results in severe crop loss. Although the pathogen grows superficially, it is associated with cytological changes within the host. This results in the exudation of teardrop like extracellular fluid from the infected host and hence the disease is commonly known as 'Wet bubble disease'.

Members of the genus *Verticillium* are generally associated with the 'Dry bubble disease' (Godfrey, 2003), cap spotting and stipe blowout disease in crops of cultivated mushrooms. The disease process involves a complex interaction between the host tissue, spores and mycelium of the pathogen. Details of these interactions are not yet clearly understood (Mills *et al.* 2003).

'Shaggy stipe disease' is caused by *Mortierella bainieri*, and is characterised by peeling of the stipe tissue of *Agaricus* mushrooms (Fletcher, 1973). The stipe and the cap are also discoloured in the course of infection. Generally a coarse grey mycelial growth similar to 'Cobweb disease' is also noticed on the infected sporocarp. The pathogen can attack mushroom sporocarps in all stages of growth. Some affected sporocarps are stunted in appearance and irregular in shape. Brown blotches are also observed on the surface of sporocarps in case of infection during the late stage of developments. The disease, although reported from some parts of England, is of little commercial importance.

Viral diseases:

The 'Mushroom bacilliform virus' causing 'La France disease' is the most well studied example of a double stranded RNA-virus causing disease in mushroom (Schisler *et al.*, 1967; Zabalgogezcoa *et al.*, 1995). Typical symptoms of this disease are cessation of sporocarp formation or malformation of sporocarps, followed by death, and also

disappearance of the mycelia from the compost bed. The disease is also known as dieback, watery stipe and brown disease of mushrooms (Godfrey, 2003).

The only other viral disease, causing considerable concern in the mushroom industries of UK, is caused by an unidentified virus X (Gaze *et al.*, 2000). Typical symptoms of this viral infection were non-productive beds of mushroom crops. The disease symptoms were reported 1996 onwards from different farms in England. The extent of disease formation appeared to be increasing each year. The variable symptoms in a typical bed affected by this virus ranged from small patches of non-productive zones in an otherwise normal cropping bed to predominant areas of no growth in the mushroom beds, with erratic growth of mushroom sporocarps in a non-productive bed. In the latter cases, the mushroom sporocarps usually appeared in clumps. In some cases, discoloured and distorted sporocarps have also been reported (Gaze *et al.*, 2000).

Bacterial diseases:

The most important bacterial pathogens of mushrooms belong to the genus *Pseudomonas*. The pseudomonad diseases in mushrooms are either associated with the formation of a characteristic blotch accompanied with a brown pigmentation or result in the formation of bacterial oozes from the infected areas. Brown blotch disease caused by *Pseudomonas tolaasii* (Moquet *et al.*, 1996), ginger blotch caused by *P. gingeri* (Wong *et al.*, 1982) and the brown discolouration of mushrooms caused by *P. agarici* (Geels *et al.*, 1994) are three distinct examples.

Symptoms of *P. tolaasii* infection are the formation of brown depressed wet lesions on the cap tissue both during growth and storage, in contrast to the *P. agarici* infection, where only the outer covering of the cap develops a characteristic brownish marble appearance and a sticky surface which can easily be removed by gently rubbing the affected area. Another disease caused by *P. agarici* is 'drippy gill' disease (Gill & Cole, 2000). Symptoms of 'drippy gill' disease include the formation of bacterial ooze on the hymeneal lamellae, which at times coalesced to form ribbons of bacterial ooze. Rainey and Cole (1988) described an unnamed disease, which had the symptoms such as malformation of the fruiting body, a characteristic constriction in the gill region, and

marked tissue degeneration with bacterial ooze in the stock (Rainey & Cole, 1988). The latter disease-causing pathogen, designated as PMS-PV29, was compared to *P. agarici* and *P. tolaasii*, and distinct differences were found. However, this pathogen could not be assigned a distinct taxonomic status.

Recently an enteric bacterium, *Ewingella americana*, has been found to be pathogenic to mushrooms. The disease is known as 'Internal stipe necrosis' (ISN) and the pathogen is associated with limited collapse of the internal stipe (stalk) tissue. This disease is also accompanied by variable browning in the centre of the stipe (Inglis *et al.*, 1996). *E. americana* is associated with the production of chitinase, in the presence of glucose and N-acetyl glucosamine, in the absence of chitin, as the strain does not utilise chitin as a carbon source. Another recently reported pathogen of brown mushroom, causing soft rot symptoms somewhat similar to *B. gladioli* pv *agaricicola*, is *Janthinobacterium agaricidamnorum* (Lincoln *et al.*, 1999). The host pathogen relation and the mechanism of disease formation have not yet been established.

P. tolaasii is associated with the production of a toxin, tolaasin. Tolaasin results in the activation of an enzyme, tyrosinase, which is responsible for the browning of the mushroom tissue (Soler-Rivas *et al.*, 1997). However, it was suggested that the production of tolaasin is not the only factor related to the formation of disease symptoms (Moquet *et al.*, 1996). *P. tolaasii* is the most discussed problem in the mushroom industry, as it has been estimated to be responsible for 8-10% of crop loss (Moquet *et al.*, 1996; Godfrey, 2003).

Several research programmes have been undertaken to look at means of preventing the tissue browning associated with these diseases. One example is the use of a cell free extract containing a lipodepsipeptide produced by *P. reactans* to inhibit browning caused by *P. tolaasii* (Soler-Rivas *et al.*, 1999). This lipodepsipeptide reacts with tolaasin, the toxin responsible for the browning of mushroom tissues. The reaction results in the precipitation of a characteristic white compound. The diffusible lipodepsipeptides produced by *P. reactans* are known as white line inducing principle (WLIP) and has been proposed as the potential inhibitor of brown blotch disease (Soler-Rivas *et al.*, 1999). Another putative bio-control strain of brown blotch bacteria was

isolated from rotting cultivated mushrooms, *Pleurotus ostreatus* and *A. bisporus* by Tsukamoto *et al.*, (1998) (Tsukamoto *et al.*, 1998). The strain 9405 is a Gram-positive, saprophytic bacterium, which does not produce tyrosinase and inhibits brown blotch symptoms *in vitro*. *In vivo* inhibition of brown blotch was not as apparent, but its candidature as a bio-control agent should be further evaluated, especially in relation to whether the strain has any adverse effects on mushrooms or humans.

1.3.5. *Burkholderia* and pathogenesis.

The genus *Burkholderia*, (Yabuchi *et al.*, 1992), consisted of three plant pathogens (*B. caryophylli*, *B. gladioli* and *B. solanacearum*), and four species known to be pathogenic to humans (*B. mallei*, *B. pseudomallei*, *B. cepacia* and *B. pickettii*). In 1995, Yabuchi and his colleagues (Yabuchi *et al.*, 1995) proposed the transfer of *B. pickettii* and *B. solanacearum* to a new genus, *Ralstonia*. Presently, the genus *Burkholderia* consists of five species, three human pathogens and two plant pathogens.

Although *B. cepacia* has long been known to be a phytopathogen responsible for soft rot of onion, it is now an increasingly recognized pathogen among immuno-compromised patients, particularly those with chronic granulomatous disease and cystic fibrosis (Govan *et al.*, 1996; Revets *et al.*, 1996). Its potential role in declining pulmonary function and other associated fatal outcome has caused widespread concern (Petrucca *et al.*, 2003; Mahenthiralingam *et al.*, 2002; Ross *et al.*, 1995; Simpson *et al.*, 1994). The most common diseases in patients with chronic granulomatous disease are pneumonia, bacteraemia, skin abscesses, and cervical adenitis. The organism's innate resistance to a number of antibiotics and disinfectants, and capacity to colonise a wide variety of nutritional substrates, means that the epidemic strains are easily transmitted within the cystic fibrosis community. The disease outcome in about 20% of such infections are generally described as a rapid decline in clinical status known as 'cepacia-syndrome', leading to septicaemia and death of individuals. Recently, a natural outbreak of subclinical mastitis caused by *B. cepacia* complex in milking sheep was reported in Spain (Berriatua *et al.*, 2001). This was the first report of *B. cepacia* infection in animals.

B. pseudomallei is commonly known as the pathogen causing melioidosis and is endemic to southern Asia and northern Australia (Dorman *et al.*, 1998). The disease varies from asymptomatic infection to fulminant sepsis. Although well known as a tropical disease, Dorman *et al.* (1998) has reported a fatal *B. pseudomallei* infection in America, in a Puerto Rican patient with X-linked chronic granulomatous disease. Other predisposing conditions that enhance the severity of melioidosis are impaired cellular immunity, pre-existing renal failure or diabetes mellitus (Schwarzmaier *et al.*, 2000).

Glanders is a contagious disease caused by the bacterium *B. mallei*, attacking hooved animals. Humans may also be infected. Horses usually develop a chronic form of the disease with infected nodules and lesions in the respiratory tract and the skin. Although this disease has been nearly eradicated from the world, it is still prevalent in some enzootic areas of Asia and Africa (Galan & Arceiz, 1997).

Of the plant pathogenic species of *Burkholderia*, *B. caryophylli* is a pathogen of carnations (*Dianthus caryophyllus*). Disease symptoms vary from stunting and wilting to cracking of stems of the infected plants (Lelliott & Stead, 1987). In Japan, *B. caryophylli* is the most damaging disease of carnations, which results in massive crop losses, especially in the warm districts of the country. The pathogen can attack almost all cultivars of *B. caryophylli*. Although the pathogen is mostly associated with *Dianthus* spp., Liu in 1990 described a similar wilting disease in *Gypsophila paniculata* and identified the causal organism as *B. caryophylli* (Liu, 1990).

Initially identified as a phytopathogen of gladiolus, several strains of *B. gladioli* pathogenic to a number of other plants as onions, iris, freessia, dendrobium, cymbidium, tulip, green gram and rice have been isolated and identified (Matsuyama, 1998). Disease symptoms vary from spotting of foliar parts to scabbing and rotting of storage tissues. Although primarily a phytopathogen, *B. gladioli* has gained much more attention since the last decade, as it has been found to cause severe pulmonary infections in cystic fibrosis and other immuno-compromised patients (Simpson *et al.*, 1994; Ross *et al.*, 1995). The expanding spectrum of human disease symptoms associated with *B. gladioli* includes bacteremia (Shin *et al.*, 1997), septicaemia and empyema (Khan *et al.*, 1996). Graves *et al.* 1997 reported the association of bacteremia, pneumonia and cervical

adenitis in immuno-compromised patients with *B. gladioli* infections (Graves *et al.*, 1997).

1.3.6. Common virulence factors identified in pathogenic *Burkholderia* spp.

Like all other pathogenic bacteria, *Burkholderia* elicit disease symptoms by production of a range of virulence factors. In theory, any molecule produced by bacteria, presented either on the cell surface or secreted to the external environment, by virtue of which bacteria colonise and destroy a specific host could act as a virulence factor (Salmond, 1994). In the case of plant pathogens, the extracellular virulence factors include plant cell wall degrading enzymes, toxins, hormones, siderophores, DNA molecules and signalling factors. The cell surface associated virulence factors are exemplified by structures like pili, flagella, lipopolysaccharides, exopolysaccharides and outer membrane bound proteins (Salmond, 1994). Over the past decade, virulence factors associated with the various members of the genus *Burkholderia* have been explored in relation to the diseases outlined above. Examples of such virulence determinants are presented below.

1.3.6a. Cell surface associated virulence factors.

Among the surface associated virulence factors, roles of flagella have been extensively studied in the two human pathogens, *B. pseudomallei* and *B. cepacia*. Chua *et al.* (2003), in their study on the aflagellate, non motile mutants of *B. pseudomallei* in the BALB/c mice model, have shown that flagella facilitate spread throughout the respiratory tract and adhesion to the host cells, but do not have any role in the invasion of host cells (Chua *et al.*, 2003). In contrast, flagella mediated motility of *B. cepacia* has been demonstrated to be the major contributory factor in the invasion of human alveolar epithelial carcinoma cell line A549 (Tomich *et al.*, 2002). The result published by Chua *et al.* (2003) contradicted previously published work of De Shazer *et al.* (1997) in which no differences could be detected in the virulence properties of non-motile aflagellate mutants of *B. pseudomallei* (1026b) in diabetic rat and Syrian Hamster models of infection. Such mixed results indicate that virulence determinants can be specific to the model system being used for the particular analysis. This was also noted by Gan *et al.*

2002, who proposed *Caenorabrhadditis elegans* as a model system for the study of melioidosis (Gan *et al.*, 2002).

In 2001, DeShazer *et al.* identified a gene for capsular polysaccharide protein in *B. pseudomallei* (1026b) with homology to glycosyltransferase gene, *wbpX*, of *Pseudomonas aeruginosa*. They demonstrated that transposon insertion in the gene renders the pathogen avirulent in the Syrian Hamster model for study of acute septicemic melioidosis (Reckseidler *et al.*, 2001). Sokol *et al.* (1999) have characterised an ornibactin biosynthesis gene, *pvdA*, which plays a significant role in the synthesis of pyoverdine type siderophores, in *B. cepacia* strain K-56, and have demonstrated its role in respiratory infection in a rat model. From their data, they suggest that the ornibactin biosynthesis and uptake plays a role in the early stages of lung colonisation in the case of *B. cepacia* infection (Sokol *et al.*, 1999).

1.3.6b. Extracellular/secreted virulence factors.

Lee and Liu (2000) identified a novel serine metalloprotease MprA, with significant sequence similarity to the subtilisin family of serine proteases, as a virulence determinant of *B. pseudomallei* (Lee, 2000). Gan *et al.* (2002) demonstrated a correlation of the virulence of clinical isolates of *B. pseudomallei* to the production of diffusible toxins by the strain, using their proposed *Caenorabrhadditis elegans* model for melioidosis. The potential toxin(s) were not identified in the report, however, their diffusible nature was established by filter assay techniques, and the gene sequence of three mutants indicated the isolation of novel proteins with significant sequence similarity to hypothetical proteins PA2762, and PA2945 of *P. aeruginosa* and a hypothetical 37.5kD protein in *agai-mtr* intergenic region of *E. coli* K-12 (Gan *et al.*, 2002).

In case of plant pathogenic *Burkholderia*, it was found that most of the strains of *B. gladioli* produced the phytotoxin toxoflavin, while the other species of the genus *Burkholderia* were associated with the production of tropolone (Iiyama *et al.*, 1998). The relationship between the production of phytotoxins and pathogenicity of *B. gladioli* in rice seedlings and onion tubers was tested in a number of strains of the pathogen.

The authors suggest that the pathogenicity of the different species of *Burkholderia* involved other unidentified factors besides phytotoxins.

Apart from the diffusion of toxins identified by Gan *et al.* (2002), export of the virulence-associated factors is generally dependent on the different secretion systems present in Gram-negative bacteria. Tomich *et al.*, (2003) used a murine model to demonstrate a direct association of type III secretion system with the virulence of *B. cepacia*. They identified the genes in *B. cepacia* with significant similarity to type III secretory proteins and demonstrated that a null mutation in *bscN* gene, encoding an ATP binding protein of the type III secretion, resulted in the attenuation of the strain (Tomich *et al.*, 2003). The *mprA* serine metalloprotease gene described by Lee and Liu (2000) has also been reported to have a signal sequence, thereby suggesting the role of *sec*-dependent transport of the mature protein (Lee & Liu, 2000). Thus, the different protein secretion systems have also been suggested to play significant roles in the pathogenesis of bacteria.

1.3.6c. Gene induction.

It is often evident that a pathogen has to reach a threshold density before expressing virulence. This is because, the regulation of genes responsible for the production of certain virulence factors depends on the concentration of signalling molecules (Lewenza *et al.*, 1999; Anand & Griffiths, 2003; Jude *et al.*, 2003), which in turn depends on the concentration of bacteria. Such density dependent induction of genes is popularly known as 'Quorum sensing'. Bacteria generally continuously produce diffusible signalling molecules at a basal level into its surrounding environment. With the increase in the quorum of bacterial cells, the concentration of such molecules in the immediate environment increases. At certain threshold concentrations, detected by specific receptors, transcriptional regulators activate specific genes. The key role in the process is thus played by the small diffusible signals well known as the 'autoinducers', which interact with their specific cognate transcriptional regulators.

The most well documented autoinducers present in the different species of bacteria are acylated homoserine lactones (Fuqua & Greenberg, 1998). A study on the distribution

of quorum sensing genes in the different members of *Burkholderia cepacia* complex has revealed the production of *N*-octanooyl-L-homoserine lactone and *N*-hexanoyl-L-homoserine lactone, in most members of the complex (Lutter *et al.*, 2001), although, presence of additional acyl-HSLs including decanoyl-HSL has been reported in *B. vietnamiensis* (Conway, 2002). These systems regulate the production of virulence factors like protease, lipase and siderophore in the different species of *Burkholderia*. The, PmlI-PmlR, and CepIR quorum sensing signals have been associated with the production of virulent metalloproteases, MprA in *B. pseudomallei* and ZmpA in *B. cenocepacia* (Sokol *et al.*, 2003; Valade *et al.*, 2004), thus indirectly implicating quorum sensing in the disease process.

1.3.7. 'Soft Rot' disease.

Cavity disease has been described as 'Soft rot' disease of mushrooms (Gill & Tsuneda, 1997). Bacterial soft rot disease in plants is loosely defined as the degradation of tissues by extracellular enzymes produced by pathogenic bacteria (Barras *et al.*, 1994). Cavity formation in mushrooms involves extensive tissue degradation of mushroom sporocarps and is hence quite naturally described as a 'Soft rot' disease of mushrooms. Depolymerisation of the cell wall components forms the main aspect of tissue maceration in the case of 'Soft rot' disease. Thus, extracellular enzymes serve as the major pathogenic factor in disease development in the case of 'Soft rot' diseases. Of the many soft rot diseases investigated in plants, studies on *Erwinia carotovora* and *Erwinia chrysanthemi* exemplify the most well documented 'Soft rot' pathogen. These species are responsible for causing tissue-macerating diseases in a variety of plants and in different plant parts (Barras *et al.*, 1994).

The virulence determinants of *Erwinia* include lipopolysaccharides (Schoonejans *et al.*, 1987), iron uptake systems (Expert & Toussaint, 1985), flagella (Mulholland *et al.*, 1993), global repressor gene *rsmA* that controls the production of extracellular enzymes, quorum sensing signals like *N*-(3-oxohexanoyl)-L-homoserine Lactone (Cui *et al.*, 1995), proteases (Martis *et al.*, 1999) and secondary metabolites (Chatterjee, *et al.* 1996). The extracellular enzymes associated with pathogenesis of these bacteria are mainly pectin degrading enzymes, besides cellulases and proteases (Barras *et al.*, 1994)

(Martis *et al.*, 1999), which together degrade the plant cell walls. Production of extracellular pectinases and cellulases has been established to be the principal pathogenic factor in the soft rot diseases (Barras *et al.*, 1994). Of the different extracellular enzymes secreted by *Erwinia* spp., the proteases are secreted by the type I secretion pathway while secretion of the range of pectin degrading iso-enzymes and cellulase depends on the general secretory pathway. Thus, the general secretory pathway has been suggested to play an essential part in the pathogenesis of the most well studied members of soft rot bacterium *Erwinia* spp.

The following section is hence dedicated to provide a brief overview of the different secretion systems found in Gram-negative bacteria, with special emphasis on the Type II secretion system.

1.3.8. Secretion systems present in Gram-negative bacteria.

Unlike eukaryotic cells, each bacterial cell can be conceived as a compartmentalised self-sufficient entity, with either a single membrane (Gram-positives) or double membranes (Gram-negatives) delimiting the cellular contents from the environment. The secretion systems in Gram-negative bacteria can be divided into two broad classes depending on the process by which proteins get exported across the inner membrane after being synthesised in the cytoplasm (Stathopoulos *et al.*, 2000; Pugsley, 1993; Wandersman, 1996; Pugsley *et al.*, 1997b; Thanassi & Hultgren, 2000).

- (i) The *sec*-dependent protein export systems, in which the secretion of proteins across the inner membrane is mediated by membrane embedded translocation complex and the export specific chaperone made up of a group of proteins called the 'sec' proteins.
 - (ii) The *sec*-independent protein export systems, in which the proteins are secreted directly across both the inner and outer membranes through special apparatus spanning both the membranes.
-

In the sequential passage of compounds across the cell membranes the most commonly used system in crossing the inner membrane is the *sec*-system, also known as the general export pathway (GEP). This is the most prevalent secretory system present in Gram-positive bacteria (Driessen *et al.*, 1998). Recently, a second secretion pathway known as the Twin-Arginine-Transport (TAT) pathway, originally identified as a protein transport route into the thylakoid of chloroplast (Hutcheon & Bolhuis, 2003), has been identified in both gram-negative and gram-positive bacteria (Berks, 2000) and members of archae bacteria (Hutcheon & Bolhuis, 2003). Proteins to be secreted undergo sorting and are directed to the respective transport apparatus in the cytoplasmic face of the bacterial inner membranes. To facilitate identification, the proteins being secreted by both the *sec*-system and the TAT pathway have N-terminal signal sequences. The *sec* pathway signal peptides are approximately 24 amino acids in length which can be divided in three distinct regions: an N-terminal positively charged region (n-region), a hydrophobic α -helical region (h-region) and a c-domain which has the signal peptidase cleavage site. The TAT pathway recognise proteins by the presence of a conserved (S/T)-R-R-x-F-L-K sequence motif at the n-region/h-region boundary of the polypeptide, with consecutive invariant arginine residues (Berks *et al.*, 2000). The other major difference between proteins that are transported by the TAT pathway and the *sec*-system is that the former transports proteins in a folded form while the later transports linear polypeptides (Berks *et al.*, 2000; Hutcheon & Bolhuis, 2003). Transport across the outer membrane is mediated by a number of systems, and each secreted protein seems to have a specific pre-destined system for its transport. Thus, the specific recognition of the secretory substrates by the specific secretin is dependent on a second group of well-defined signals as outlined below.

As presented in figure 1.3., secretion systems in Gram-negative bacteria will be divided into two broad classes in this review, essentially categorised by their dependence on the *sec*-system for transport of proteins across the inner membrane.

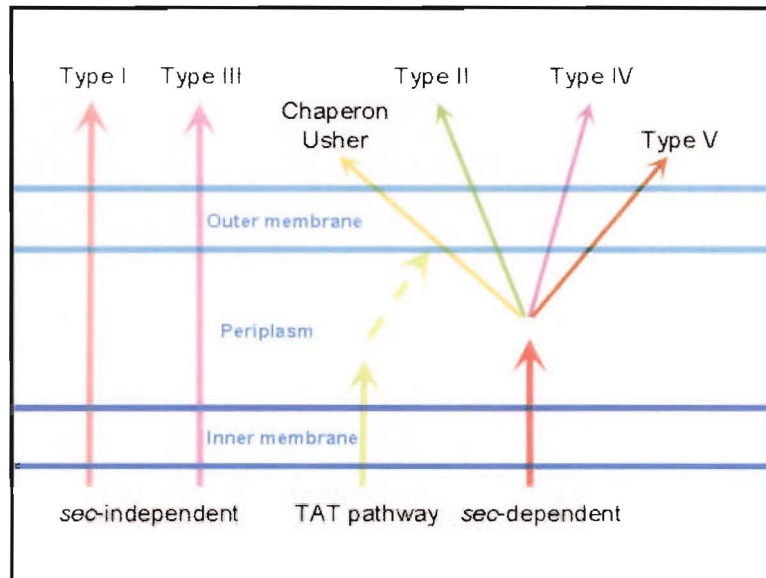


Figure 1.3: General overview of the different secretory systems present in Gram-negative bacteria.

1.3.8a. The *sec*-dependent secretory systems:

The four types of secretion pathways, which facilitate transport from the periplasm to the external milieu, are categorised as:

- (i) Type II secretion: responsible for the secretion of extra cellular enzymes and toxins.
- (ii) Type IV secretion: a system adapted to function in the transport of diverse substrates such as DNA in bacterial conjugation and T-DNA into the plant cells and delivery of a variety of effector proteins produced by pathogenic bacteria into the target eukaryotic cells.
- (iii) Type V secretion: secretes proteins with diverse functionalities as proteases, toxins, adhesions and invasions.
- (iv) Chaperone /Usher Pathway: mainly responsible for the secretion of the different kinds of pili that enable adhesion of pathogenic bacteria to the respective host cell types.

Type II secretion system:

The type two-secretion system is the main terminal branch of two-step secretion processes. It generally consists of 12 proteins spanning the cell envelope to form the core of the secretin, besides some associated proteins present in specific cases for the efficient functioning of the secretory machinery (Peabody *et al.*, 2003). The type two secretion apparatus has been studied and identified in a range of Gram-negative bacteria including *B. cepacia* (Kimoto & Nakazawa, 2000; Fehlner-Gardiner *et al.*, 2002) and *B. pseudomallei* (DeShazer *et al.*, 1999), and is popularly known as the General Secretory Pathway (GSP) of protein secretion. Most type II systems secrete more than one structurally divergent protein through the same set of secretion machinery (Pugsley *et al.*, 1997b). An exception is *Klebsiella oxytosa*, which secretes only one enzyme, pullulanase (Pugsley *et al.*, 1997a).

The different components of the type II secretion system consist of GspD (Figure 1.4), which is the only outer membrane associated protein forming the secretin in the outer membrane. Proteins GspG, H, I, J have periplasmic domains and major similarities to type IV pili proteins for which they are also known as the pseudopilins (Peabody *et al.*, 2003). Another subset of inner membrane bound proteins is present, (GspC, F, K, L, M, N) which also have periplasmic domains but do not show any sequence similarities to the pili proteins. GspE is the only cytoplasmic protein, and possesses a conserved ATP-binding motif thereby providing an autokinase activity; evidently it energises the secretion process or assembly of the secretory apparatus (Thanassi & Hultgren, 2000). Apart from these, two other GSP proteins are often found in the systems and known as GspM and GspL (Peabody *et al.*, 2003). The latter is known to be the responsible for the association of GspE to the membrane, while the former is known to be the stabiliser for GspE. In some cases, a periplasmic protein GspS is also found, and is believed to act as a molecular chaperone in guiding the secretory substrates to the secretin, GspD.

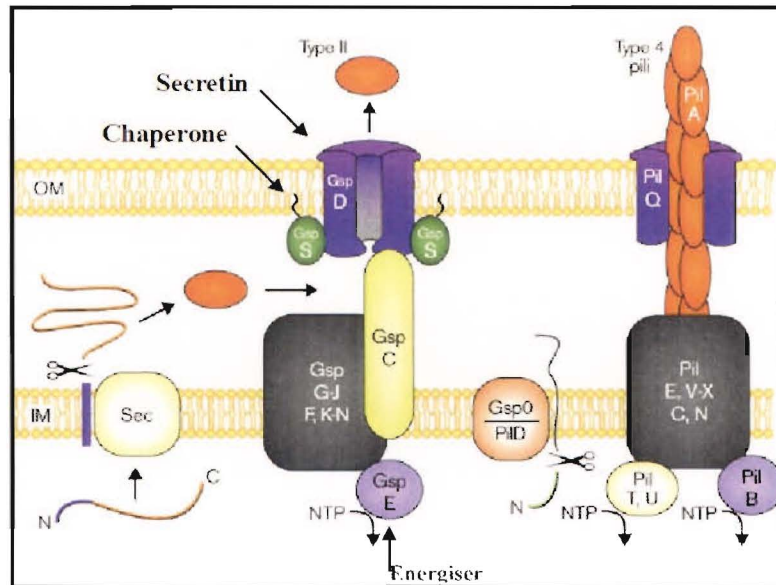


Figure 1.4: Diagrammatic representations of Type II and Type IV secretion systems found in gram-negative bacteria (Thanassi & Hultgren, 2000).

The GSPs in the different bacterial systems have distinct signals, which are recognised specifically by the outer membrane pore forming protein GspD, although no specific domains on the secreted polypeptides could be designated as a signal sequence in any of the secreted products. However, specific relationship between the folding of polypeptides in the periplasmic space (Nakazawa & Mitsuko, 1996; Stathopoulos *et al.*, 2000) and their ability to be secreted across the outer membranes has been reported in many cases (Pugsley *et al.*, 1997b). It is envisaged that the accurate folding of polypeptides expose certain motifs which are recognised by the chaperones and act as the signals necessary for the recognition of candidate proteins to be secreted by the type II machinery and moved across the outer membrane of the cell (Stathopoulos *et al.*, 2000).

The secretion machineries in the different systems studied shows a high species specificity and generally cannot functionally complement *gsp* operon of a closely related species (Pugsley *et al.*, 1997b). However, deGroot *et al.* (2001) have recently shown that the recognition of substrates by the GSPs of two closely related species can be exchanged (de Groot *et al.*, 2001). The pullulanase secretion of *Klebsiella oxytosa*,

was the first studied type II secretion apparatus and is considered to the prototype of the GSP in Gram-negative bacteria (Pugsley *et al.*, 1997a).

Pullulanase, product of *pulA* gene, is a 120kD oligomeric lipoprotein that is secreted by *K. oxytosa* only when grown in media containing starch, maltose or intermediate size dextrans (Pugsley *et al.*, 1997a). The enzyme cleaves α -1,6 linkages in maltodextrin polymers thereby liberating linear dextrans, which can then be utilised by the bacterial cells. Secreted PulA is temporarily anchored to the cell surface of the bacteria by the N-terminal fatty acid moiety before being released into the media. The secretion of PulA requires 14 specific gene products, which form the 'pullulanase secreton' and the DsbA protein (Stathopoulos *et al.*, 2000). The secreton is made up of a cytoplasmic protein, PulE, which consist of an ATP binding site and a tetracysteine motif (Possot & Pugsley, 1997), and functions as the potential energiser of the system. The integral inner membrane proteins PulC, PulM, PulN, PulK and PulF form the basal body of the secretion apparatus and are anchored to the periplasmic side of the inner membrane. They are generally attached to the inner membrane by a single N-terminal domain followed by a large C-terminal domain. PulC is suggested to form a bridge between the components situated in the two membranes. The four pilin like proteins PulG,H,I and J possess prepilin peptidase cleavage and methylation sites, localised in the periplasm and has been suggested to form a secretion channel for the passage of PulA (Stathopoulos, 2000). In addition to these four prepilin peptidases, the pullulanase secretion system consists of a pilin-like signal peptidase, which is an absolute necessity for the secretion of pullulanase (Pugsley *et al.*, 1997a). PulD forms the outer membrane bound secreton consisting of 10-14 multimers spanning the membrane. PulS serves as a molecular chaperone for the pullulanase secretion system and is responsible for the targeting of PulA to the PulD secretin by interaction with the C-terminal domain. The N-terminal domain of PulD is suggested to be responsible for the substrate recognition and also interaction of PulC. Pull is a monotopic inner membrane bound protein that interacts with the energiser molecule of the system, PulE (Stathopoulos *et al.*, 2000).

Possot and Pugsley (1997) have shown that the tetracysteine motif of PulE is essential for the effective functioning of the secreton in the pullulanase secretion. They have also compared the PulE homologues present in a number of other Gram-negative bacteria,

EpsE of *V.cholerae*, OutE of *E. chrysanthemi*, XcpE in *P. aeruginosa* and XpsE in *Xanthomonas campestris*. With the exception of *Xanthomonas*, all the PulE homologues investigated had the 4 cysteine motif (Possot & Pugsley, 1997).

In 1996, Sauvonnet and Pugsley demonstrated that the two non-adjacent regions at the N-terminal region of PulA, were together necessary and sufficient for the efficient secretion of the pullulanase. Absence of either one of the regions reduced the secretion of pullulanase and secretion was completely abolished in the absence of both. They created a series of deletion strains from the C-terminal end of the pullulanase gene, fused β -lactam to each of the truncated genes and monitored the secretion of BlaM. Two regions, were identified as A and B, which were 78 and 80 amino acids in length and present in the N-terminal end that served as the secretion signal for PulA (Sauvonnet & Pugsley, 1996). Lu and Lory 1996 applied a similar β -lactam fusion approach to the exotoxin A of *P. aeruginosa* and identified a 60 amino acid signal sequence at the N-terminal region of toxin A (Pugsley *et al.*, 1997b).

GSP apparatus in different Gram-negative bacteria have been found to consist of homologues to the Pul proteins. In *P. aeruginosa*, it is known as the 'xcp gene' cluster and is associated in the secretion of toxin A (Mc Vay & Hamood, 1995), elastase, LasA, alkaline phosphatase, lipases and phospholipaseC (Sandkvist, 2001). In members of *Erwinia* spp it is known as the 'out' gene cluster and is associated with the secretion of pectinases, cellulase (Barras *et al.*, 1994) and polygalacturonase (Sandkvist, 2001). In *Vibrio cholerae* it is known as the 'eps' system and is responsible for the secretion of the cholera toxin (Sandkvist *et al.*, 1997), neuraminidase, lipase (Sandkvist, 2001), protease as well as an endochitinase (Connell *et al.*, 1998). In *Xanthomonas campestris*, the cluster is known as the 'xps' gene cluster and is associated with the secretion of polygalacturonate lyase, α -amylase, protease and endogluconase (Sandkvist, 2001). In *Aeromonas hydrophila* the general secretory pathway is designated as the 'exe' and is involved in the secretion of toxin, aerolysin, amylase, phospholipaseC, proteases and DNase (Sandkvist, 2001) and cell surface structures (Howard & Meiklejohn, 1995). Generally, the genes of the pathway are clustered in one single region of the chromosome (Pugsley *et al.*, 1997b). The *gsp* gene cluster has recently been found to be present in *E. coli* K-12 and has been associated with the secretion of endogenous

extracellular protein ChiA (Francetic *et al.*, 2000). DNA sequence analyses of the proteins constituting the GSP secretion machinery (Figure 1.4.) in the different species of bacteria studied so far reveal 30-60% identity at the protein level. Most of the genes in the *gsp* gene cluster of the different species are organised in a single operon with an exception observed in case of *P. aeruginosa*, (Figure 1.5) which has two diverging operons. Minor variations in the organisation of the genes are sometimes observed at the 5' or the 3' termini of the gene cluster (Sandkvist, 2001). The *gspC* gene in *B.pseudomallei* 1026b *gsp* operon is transcribed in a direction opposite to the rest of the genes (De Shazer *et al.*, 1999).

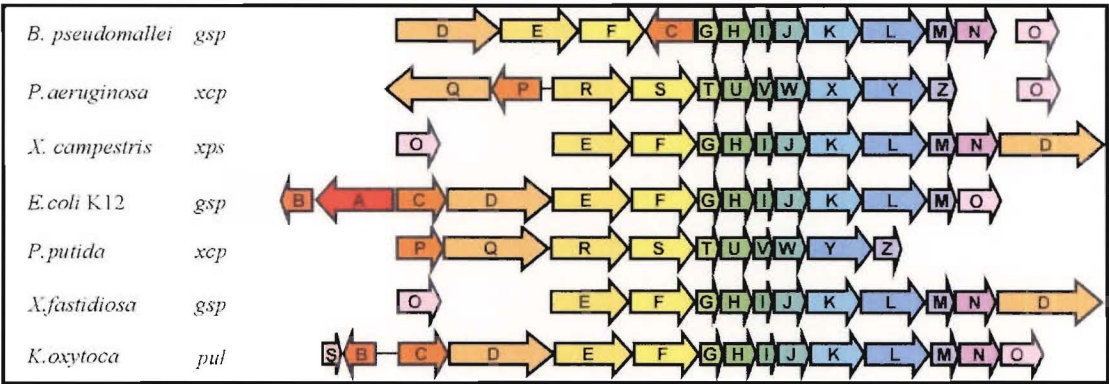


Figure 1.5: Alignments of the genes encoding type II secretion pathways studied in some bacteria. The individual species with the names of their secretion genes are presented on the left side. The figure represents a schematic diagram of the genes from A to O, each colour represents a specific gene and its homologue in case of *P. aeruginosa*.(Figure adopted from Sandkvist, 2001)

The *gsp* gene cluster in *E. coli* K-12 has been designated ‘cryptic’ (Francetic & Pugsley, 1996) as under normal laboratory conditions the strain does not secrete any proteins to the extracellular media. As a result, initially, the system could not be traced in *E. coli* K-12 for a long time. The first indication of the system being present in *E. coli* came from partial sequence analysis of a protein which had homology to the MTB protein O, and the entire operon was identified following the sequencing of the *E. coli* K-12 chromosome (Francetic & Pugsley, 1996). Sequence data reveal the presence of a full complement of the *pul* genes, *pulC-O*, present in *E. coli* K-12, namely *gspC-O*. Generally, the secretion apparatus in most of the members of Gram-negative bacteria consist of all the proteins stated in case of pullulanase secretion. However occasionally,

some proteins may be present, an example is the absence of the *xcpK* (*gspK*) homologue in type II system of *P. putida* (de Groot *et al.*, 1999).

Type IV secretion system:

The type IV secretion system is homologous to the *virB* system in *A. tumefaciens* dedicated to the transport of oncogenic T-DNA into plant cells (Dang *et al.*, 1999; Seubert *et al.*, 2003). Examples of protein secretion by this machinery are CagA protein secretion by *Helicobacter pylori* into the infected gastric epithelial cells, secretion of multicomponent *Bordetella pertussis* toxin secreted by the Ptl system (Seubert *et al.*, 2003) and secretion of a toxin by *Legionella icm/dot* involved in phagosome trafficking and macrophage killing while residing in the eukaryotic vacuoles (Dang *et al.*, 1999). Although the best studied secretion apparatus in case of type IV system is the *virB* systems of *Agrobacterium*, Figure 1.4. represents the *P. aeruginosa* type IV pili synthesis system, to depict the degree of similarity to the type II secretion apparatus (Thanassi & Hultgren, 2000).

The type IV pili or the secretion apparatus are polarly localised and play an important part as the virulence determinants in bacteria, by enhancing adherence to the target cells and also mediating a specific kind of motility known as twitching motility (Stathopoulos, 2000). Secretion and assembly of the type IV apparatus also require 14 proteins, some of which have similarities to the type II systems (Stathopoulos *et al.*, 2000). In the case of type IV pili, the pili subunits get cleaved off the signal sequence at the cytoplasmic face of the inner membrane, in contrast to that of the periplasmic face in case of all the other mentioned systems (Stathopoulos *et al.*, 2000). Translocation of the pili subunits to the membrane surfaces in case of *P. aeruginosa* and *N. gonorrhoeae* is facilitated by PilQ, which forms the secretion channel. However, in case of *virB* system, no such outer membrane associated secretin is found (Thanassi and Hultgren, 2000). In *P. aeruginosa* and *N. gonorrhoeae* there are three energy transducer systems present in the cytoplasmic face. The first one is Pil B, a mutation in which results in the loss of surface pili; the other two are Pil T and Pil U, mutations in either one of which results in hyper-piliated strains, although they still remain devoid of the capacity of twitching motility (Alm & Mattick, 1997).

Type V secretion system:

Some proteins get transported across the outer membrane either with the help of certain domains present as a part of their structures (known as the autotransporter family) or, aided by helper protein(s) (consisting of two types, the two-partner systems and Oligomeric coiled coils adhesin or the Oca Family) (Desvaux *et al.*, 2004). Together, these systems are categorised as the Type V secretion system. The self promoted extra cellular secretion system found in *Neisseria* and *Haemophilus* for the secretion of immunoglobulin A protease is generally taken as the prototype for the autotransporter secretion pathway. Examples of the two-partner system are extracellular release of the mature filamentous haemagglutinin (FHA) of *B. petrusis* and, *Serratia marcescens* hemolysin secretion system. While the prototype of Oca family of secretin is the YadA secretion in *Yersinia enterocolitica* (Desvaux *et al.*, 2004).

The characteristic feature of proteins secreted by the autotransporter system is the presence of a few distinct domains in the periplasmic precursor polypeptides, other than the N-terminal signal sequence. These domains help in either the recognition of the helper proteins or in the passage of the proteins across the outer membrane after the polypeptides are autocatalytically cleaved off their signal sequence at the periplasmic face of the inner membrane (Thanassi & Hultgren, 2000). In contrast, in case of the twine partner system, the exoprotein and the β -barrel forming proteins are separately translated as two distinct proteins, while the members of the Oca family have six different domains, as exemplified by in the YadA secretion in *Y. enterocolitica* (Desvaux *et al.*, 2004).

The *Neisseria* and *Haemophilus* immunoglobulin A protease splits the hinge region of the human IgA (Wandersman, 1996). Such proteins that support self-transport are characterised by the presence of a distinct C-terminal domain, which enables them to anchor themselves in the outer membrane and then gradually get secreted through a barrel-like pore formed by the β barrel domain of the protein (Figure1.6B left). The mature protein, in this case, gets folded into the secondary structure before being secreted into the external milieu, and is auto-proteolytically cleaved off the C-terminal sequence after being docked into the periplasmic face of the outer membrane. The other

accessory domains (α and γ) also get cleaved off before the mature protein is liberated into the external environment (Desvaux *et al.*, 2004).

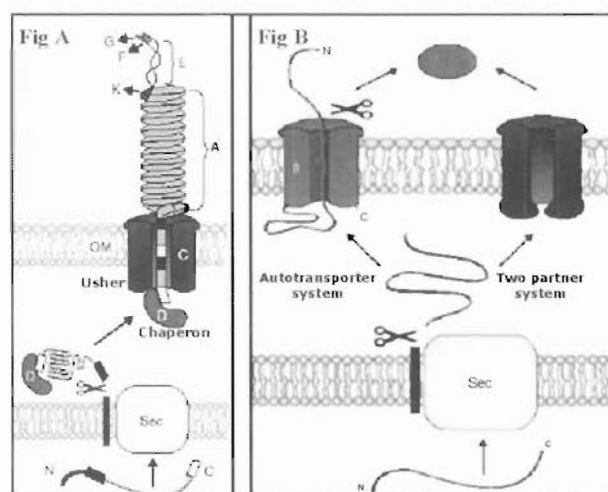


Figure 1.6: Diagrammatic representations. [A] Chaperon Usher pathway (The alphabets representing the respective Pap proteins of the P pili). [B] Type V secretion systems present in Gram-negative bacteria (Thanassi & Hultgren, 2000).

The *S. marcescens* pore forming hemolysin, ShlA is secreted across the outer membrane in the presence of a single separate helper protein, ShlB. The activation of ShlA depends on its interaction with the helper protein. ShlB not only facilitates secretion across the outer membrane, but also helps in processing of the precursor protein. The site of interaction of these two proteins lies in the N-terminal region of ShlA. Cleavage of 149 amino acid residues from the N-terminal end of ShlA protein renders it inactive and incapable of being activated by ShlB, thereby suggesting the presence of an activation domain of ShlB in ShlA (Wandersman, 1996). *Proteus mirabilis* haemolysin and *B. petrusis* FhaB proteins have similar secretory systems (Thanassi & Hultgren, 2000).

Chaperon/Usher Pathway:

The production of P pili and the type I pili are good examples to illustrate the chaperon/usher pathway, which is an alternative terminal branch of protein secretion found in Gram-negative bacteria (Stathopoulos *et al.*, 2000). The components of the secretion machinery is characterised by the presence of a periplasmic chaperon, which

plays multifunctional roles of facilitating the release of the polypeptides from the *sec* apparatus, enabling the proper folding of the periplasmic intermediate, and the guided movement of the secretory protein to the inner face of the usher, or the secretin on the outer membrane (Thanassi & Hultgren, 2000). As represented in Figure 1.6A., PapD plays the role of the periplasmic chaperone, while PapC is the outer membrane usher, forming a ring shaped multimeric component on the membrane surface (Thanassi *et al.*, 1998). Apart from the chaperon and the usher, the system also requires the products of six genes - PapA, the major pilin; PapE, the minor pilin and generally present at the tip of the composite pili; PapG, which forms the adhesin and is involved in the recognition of the Gal α moieties of the kidney cells (Thanassi *et al.*, 1998); PapF which forms the adapter moiety of the PapG to the PapE; and PapK, products of which link the minor pili fibrils to the PapA rod. The rod is terminated by PapH, which also plays the role of an anchor to the membrane. The chaperon directs the polypeptides to the secretin and once the substrates reach the usher, PapC, the chaperone, dissociates. The pathway does not require input of external energy for the secretion of proteins across the outer membrane, and the movement of the pilin subunits are supposed to take place by diffusion across the usher (Stathopoulos *et al.*, 2000).

1.3.8b. The *sec*-independent secretory systems.

The ABC transport systems and the type III secretion system, which have major structural similarities to the flagella apparatus, exemplify the *sec*-independent protein transport systems. In both these cases the proteins get transported directly into the external surface without the formation of any periplasmic intermediate.

ABC Transport System

The ABC transporters are associated with the transport of a wide variety of substrates from the cell to external environment in both eukaryotes and prokaryotes. Examples of proteins transported by the ABC transport system are include α -haemolysin by *E. coli*, metalloproteases by *E. chrysanthemi* and *S. marcescens*, alkaline protease by *P. aureginosa*, hemoprotein (HasA) by *S. marcescens*, leucotoxin by *Pasteurella hemolytica*, toxin adenyl cyclase (CyaA) by *B. petrusis* (Wandersman, 1992) and

Colicin V by *E. coli* (Zhang *et al.*, 1995). In gram-negative bacteria, the ABC proteins, in conjunction with other proteins are also involved in the uptake of certain specific substrates like maltose, histidine, iron chelating siderophore and oligopeptide complexes (Wandersman, 1996).

The main characteristic of this system is the presence of a membrane bound ATPase as an inner membrane component of the secretion apparatus. Essential characteristics of ATPases are the presence of a hydrophobic domain, which spans the membrane six times, and a nucleotide binding cytoplasmic domain. The cytoplasmic domain forms a fairly conserved cassette while the hydrophobic domains are not conserved. In general, the secretion signal of the proteins secreted by the ABC transporters is situated close to the C-terminal end of the polypeptides being secreted (Thanassi & Hultgren, 2000). However, an exception to this is found in case of Colicin V secretion in *E. coli* which has an amino terminal secretion system (Zhang *et al.*, 1995).

The first protein exporter system identified was the α -hemolysin transporter of *E. coli* and is presented in Figure 1.7. The secretory apparatus consists of three components. The inner membrane bound ABC transporter, the inner membrane fusion protein or the MFP and the outer membrane protein (OMP), which in case of hemolysin transport is TolC. The outer membrane protein forms a barrel that spans the outer membrane and extends into the periplasm. The membrane fusion protein is generally trimeric and traverses the periplasmic space linking the OMP to the ABC transporter. The ABC protein having the nucleotide-binding site acts as the energizer for the whole system (Thanassi & Hultgren, 2000).

Generally, the ABC transporters are associated with the export of either one specific protein or with the secretion of a group of isoenzymes, thereby indicating high specificity. However, the secretion systems of *E. coli* hemolysin, *E. chrysanthemi* and *P. aeruginosa* metalloproteases have been reconstituted in *E. coli*.

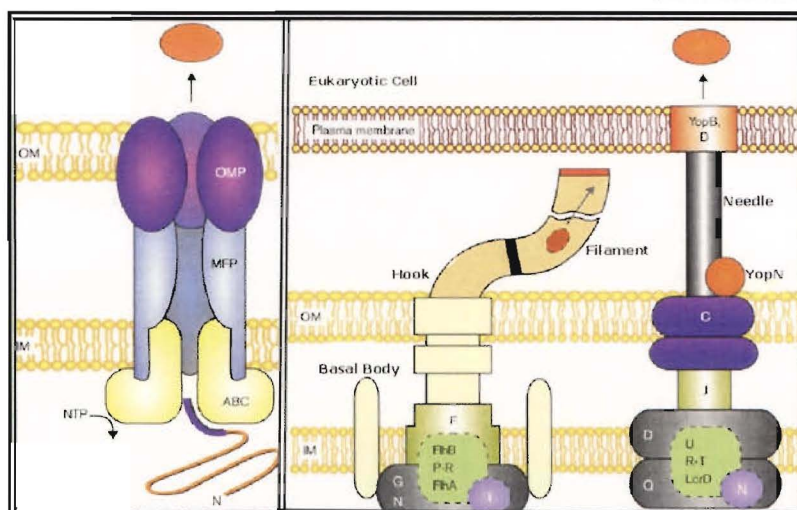


Figure 1.7: Diagrammatic representations of Type I secretion system (Left) and Type III secretion system of *Yersinia* sp. (Right). (Thanassi & Hultgren, 2000)

Type III secretion:

The type III secretion apparatus is the most sophisticated system that connects secretion to pathogenesis (Cornelis & Van Gijsegem, 2000). It generally enables the pathogenic bacteria adhering to the surface of the eukaryotic cells to inject bacterial proteins into the host cells across three membranes, two present in bacteria and the host cell membrane. The system consists of a secretion apparatus and an assortment of secreted proteins, some of which act as effectors by directly affecting the target cells, while others are translocators that enable the passage of effectors across the eukaryotic cell membranes. Type III secretory systems are present both in animal and plant pathogens. In the latter case, they are responsible for elicitation of disease or hypersensitive reactions. The effector proteins in plant pathogens belong to two classes, harpins and avirulence proteins. The well known animal pathogens exhibiting type III system mediated diseases are *Yersinia*, *Shigella*, *Salmonella*, *Bordetella*, *Pseudomonas* (Thanassi & Hultgren, 2000), *Burkholderia cepacia* (Tomich *et al.*, 2003), enteropathogenic and enterohemorrhagic *E.coli* and express diseases ranging from fatal septicaemia to mild diarrhoea and from fulgulant diarrhoea to chronic lung infection (Cornelis & Van Gijsegem, 2000). Well-documented examples of plant pathogenic bacteria exhibiting disease through type III secretion system include *Erwinia amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas* spp.

The *Yersinia* type III secretion machinery was the first studied apparatus and still remains the classic example for the system. The *Yersinia ysc* operon consists of 29 genes involved in the secretion of the *Yersinia* outer membrane proteins or the Yops. 11 out of the 29 *ysc* genes have homologues in most of the other type III systems. The *yscC* gene product is a member of the secretin group of proteins (Figure 1.7) and is responsible for the formation of the outer membrane bound ring shaped structure facilitating the passage of macromolecules and filamentous phages. The four Ysc proteins, YscD, YscR, YscU and YscV span the inner membrane. YscS and YscT have also been predicted to be inner membrane bound. The energiser for the system is YscN, which has the ATP binding motifs. YscJ is a lipoprotein and has not been localised in the *Yersinia* secretion apparatus, but its counterpart in *Pseudomonas* spans across the inner and the outer membranes. The other two conserved proteins in the type III system are YscL and Q. Amongst the non conserved proteins in the system are YscW, which helps in the proper insertion of YscC proteins in the outer membrane and the YscO and P proteins which are released upon calcium chelation, and is thus suggested to belong to the external part of the apparatus (Cornelis & Van Gijsegem, 2000).

The well-known effectors in *Yersinia* are YopH and YopE proteins (Wandersman, 1996). Both serve as cytotoxins, with the former exhibiting a tyrosine phosphatase activity, while the latter is involved in the disruption of the actin microfilament structure. The translocation of the YopE and YopH require their secretion and also the secretion of YopB and YopD proteins, which act as the translocators for the system. The YopB and YopD proteins have hydrophobic domains, thereby suggesting a transmembrane location (Cornelis & Van Gijsegem, 2000).

1.4. Rationale for investigating this disease mechanism.

Mushrooms have a characteristic cell wall composition (section 1.3.3) distinct to plants and animals, in which *Burkholderia* has previously been shown to cause disease. The initial characterisation of the pathogen has shown that it causes deep pitting of the sporocarp tissue. This suggests a rapid disintegration of the fungal mycelia aggregated in the formation of mushroom sporocarps. Another interesting aspect of the disease is that pitting of the sporocarp tissue is not associated with tissue browning.

The ability of the genus *Burkholderia* to colonise both animal and plant tissues has made it an interesting organism to study in relation to host pathogen interactions. Different strains of *Burkholderia* have been found to be associated with the degradation of a variety of chlorinated hydrocarbon compounds (Shields *et al.*, 1995; Dubras *et al.*, 1996). *P. gladioli* B-1 is a strain that has the ability to form and cleave C-P bonds (Nakashita & Seto, 1991). It has been demonstrated to cleave the C-P bond of 2-amino-3-phosphopropanoic acid while the ability to produce the enzyme phosphoenolpyruvate phosphomutase provides it with the capacity to form C-P bonds (Nakashita *et al.*, 1992).

Species of the genus *Burkholderia* possess multiple chromosomes and a very interesting genomic organisation, consisting of an array of insertion sequences (Lessie *et al.*, 1996). It has been suggested that these insertion sequences promote the genomic variability of the genus. Some species of the genus *Burkholderia* have also been found to harbour a considerable amount of extrachromosomal DNA elements, including a number of transposons. Some of these transposons carry genes that are responsible for the degradation of chlorinated hydrocarbons (Xia *et al.*, 1998). Insertion elements in the genome of *Burkholderia* have also been found to be associated with the activation of various operons, at different times, thereby leading to the capacity of the genus to metabolise a wide range of hydrocarbons (Johnson & Ronald, 1997). *Burkholderia* species have also been reported to possess multiple pathways for the direct metabolism of the catechols (Johnson & Ronald, 1997). In an attempt to establish the role of the different phytotoxins in the pathogenesis of *Burkholderia* spp., Iiyama-Kazuhiro *et al.*, 1998 (Iiyama *et al.*, 1998) have found that species of *Burkholderia* are associated with

the production of tropolone and toxoflavin. Tropolone is a potent tyrosinase inhibitor and inhibits the enzyme by a so-called non-classical mechanism, where by the enzyme-inhibitor complex undergoes a relatively slow reversible reaction (Ferrar & Walker, 1999).

Taking into consideration all these issues, it was hypothesised that *Burkholderia gladioli* pv *agaricicola* produced some kind of inhibitor of mushroom tyrosinase, followed by the production of cell wall degrading enzymes. It is possible that the inhibitory compounds and the cell wall degenerating compounds are either produced and function separately or have a synergistic effect, thereby causing the massive degradation of the tissue. Initial studies with *B. gladioli* have also revealed that the microorganism inhibits a range of other phytopathogenic filamentous fungi, yeast and the anaerobic bacterium *Lactobacillus*. This suggests that the strain is associated with the production of either more than one toxic compound or a broad-spectrum toxin. In addition, the strain has been reported to be causing acidification of the media. This could also be a reason for inhibition of tyrosinase. It was predicted that at least two groups of compounds are involved in the pathogenesis of the species, one of which paralyses the host's defence mechanism and the other then starts the degradation of the mushroom mycelia, there-by causing the pitting. Thus, a wide range of possibilities arises by which BG164R causes Cavity disease in white button mushrooms. This project was hence initiated to try and elucidate the mechanism of disease formation by identifying the genes involved in the process.

1.5. Thesis objectives and aims.

Cavity disease appears sporadically, but poses a massive threat to the mushroom industry. Previous work has not significantly advanced our understanding of how BG164 causes Cavity disease. This thesis is hence the first attempt to define the genetics of virulence and propose probable models involving the sequential stages of Cavity disease formation by BG164. The presented research was initiated with two broad objectives:

Objective1: To identify the gene(s) of BG164R involved in the expression of Cavity disease of white button mushrooms and to identify the mechanism(s) by which they cause the rapid degradation of mushroom tissues.

Objective 2: To study the host pathogen relationships of *B. gladioli* pv. *agaricicola*, involved in the expression of Cavity disease in *Agaricus bisporus*.

To address the two objectives several aims were set at the outset of the project.

Objective 1 aims:

- 1) Generate antibiotic resistance profile of BG164 and identify/create a marker that would enable us to identify the strain in the gene manipulation experiments.
 - 2) Establish an efficient mutagenesis system for BG164.
 - 3) Isolate virulence genes using a novel mushroom bioassay.
 - 4) Identify the nature of the gene product(s) involved in the initiation of the disease.
 - 5) Investigate whether the antifungal compound(s) produced by BG164 is involved in the degradation of mushroom mycelia.
-

Objective 2 aims:

- 1) Identify the endogenous microbial population prevalent in button mushrooms.
- 2) Define the minimum infectious unit of the pathogen required to initiate Cavity disease in button mushroom.
- 3) Find out whether initiation of the disease process is dependent on the presence of inducing signals, as an attempt to identify a reason for the sporadic appearance of the disease.

1.6. Presentation overview.

The results presented in this thesis have been divided into six chapters. The present Chapter, has broadly introduced the host and the pathogen involved in this study, and, provided a general idea about the different mechanisms by which Gram-negative bacteria cause disease.

The first part of Chapter Two describes the establishment of an efficient bioassay system and the isolation of a spontaneous rifampicin resistant mutant strain of the pathogen. These two essential components were used subsequently in the genetic analyses of the disease causing genes of *B. gladioli* pv. *agaricola*, strain BG164. Molecular experiments aimed at targeting, identifying and isolating the genes of BG164R responsible for the formation of cavity disease are also described in Chapters Two, Three and Four. Preliminary biochemical experiments attempted to co-relate the genetic findings to the biological experiments has been described in Chapter Three. Occasional connections with the biological work have been made throughout the thesis, since biological assays formed guidelines to genetic manipulations and in some cases play an important role in confirming data obtained by the molecular experiments.

The second part of the thesis, Chapter Five, addresses some biological questions and describes experiments carried out purely on the basis of host pathogen relationships. Their probable interactions appear to play a vital part in the initiation of disease, as evidenced from the data presented.

In the concluding chapter, Chapter Six, results of the key findings in the project are summarised, probable models describing the mechanism(s) by which BG164R causes cavity disease in button mushrooms are suggested and ways to test the models are proposed.

Chapter II

Isolating avirulent mutants of BG164R.

2.1. Introduction.

Bacterial virulence arises from a wide range of biochemical and physiological processes taking place within the pathogen. The capacity to cause disease is ultimately based on the sequential regulation and expression of gene(s). The biggest challenge in studying host pathogen relationships is to identify the precise order in which virulence factors are regulated. Identification of the potential pathogenic genes, irrespective of the temporal nature of their expression, forms the preliminary basis of studying host-pathogen interactions. Such investigations are generally approached by creation and identification of null mutations that knock out the virulence properties, followed by the establishment of Molecular Koch's (Falkow, 1988) postulate which involves isolating and restoring the virulence properties by an intact copy of the knocked out gene. This fundamental approach forms the basis of studying the genetic mechanism(s) of Cavity disease formation by BG164 in this study.

Both targeted gene mutations and random mutagenesis can be used to identify putative virulence genes. When predetermined genes with well-defined phenotypes are targeted, site-directed mutagenesis and chemical mutagenesis are useful. However, when identification of a gene is based on tracing the position of the specific mutation following random mutagenesis of the bacterial chromosome, transposons are generally the method of choice. Transposons provide the primary advantage of encoding an easily followed selectable marker, which allows identification of the mutated genes. In the previous reports on Cavity disease, propositions about the possible gene products involved in the pathogenesis of BG164 have been put forward by Gill and Tsuneda (1997). Their list may not be comprehensive, so in this study a new search was initiated using random (transposon) mutagenesis.

This chapter mainly describes an attempt to target the genes of *B. gladioli* pv. *agaricicola*, involved in causing cavity disease in mushrooms. At the beginning, this required construction of strains to be used in the analyses. Random transposon mutagenesis using mini-Tn5KmlacZ2 (deLorenzo *et al.*, 1990) was used to introduce the mutations. The chapter also describes phenotypic characterisation of the mutants and discusses the results in light of the objectives of this research and also in relation to other research involving host pathogen interactions.

2.2. Material and Methods.

2.2.1. Bacterial strains and plasmids used.

Strains and plasmids	Genotype or description	References
<i>Burkholderia gladioli</i> pv. <i>agaricicola</i> .		
BG164	wildtype, cav+, Af+, Rif ^S	Gill <i>et al.</i> 1997
BG164R	wildtype, cav+, Af+, Rif ^R , Prot+	This study
BG4-12	BG164R <i>gspF</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG12-88	BG164R <i>gspK</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG12-147	BG164R <i>gspK</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG15-40	BG164R <i>gspE</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG15-87	BG164R <i>gspD</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study

BG16-787	BG164R <i>gspE</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Aft+, Prot-	This study
BG4-127	BG164R::mini-Tn5KmlacZ2,Rif ^R ,Km ^R ,cav-	This study
BG9-130	BG164R::mini-Tn5KmlacZ2,Rif ^R ,Km ^R ,cav-	This study
BG9-481	BG164R::mini-Tn5KmlacZ2,Rif ^R ,Km ^R ,cav-	This study
BG14-48	BG164R::mini-Tn5KmlacZ2,Rif ^R ,Km ^R ,cav-	This study
BG14-127	BG164R::mini-Tn5KmlacZ2,Rif ^R ,Km ^R ,cav-	This study
BG14-722	BG164R::mini-Tn5KmlacZ2,Rif ^R ,Km ^R ,cav+, Prot+	This study
BG110	BG164R::mini-Tn5KmlacZ2,Rif ^R ,Km ^R ,cav+, Af ^R	This study
<i>Escherichia coli.</i>		
S17-1(λpir)	<i>thi pro hsdR- hsdM+ ΔrecA</i> RP4-2::TcMu- Km::Tn7	Simon <i>et al.</i> 1983
MC4100	F ⁻ <i>araD139Δ(lacI PO ZYA-argF)</i> U169 <i>rpsL</i> <i>thi recA-56</i>	Casadaban 1976
<i>Pseudomonas aureofaciens</i>		
PA147-2	wildtype, Af+, Bfm+, Rif ^R , Cm ^R , (10μg/mL)	Carruthers <i>et al.</i> 1994
PAE639	PA147-2 <i>yeiJ</i> ::mini-Tn5KmlacZ2,Af+,Bfm-, Fla-, Mot-, Rif ^R , Km ^R	Monds MSc Thesis 2000
<i>Pseudomonas cepacia.</i>		
B111	wild type, Chi-	A.L.J.Cole
<i>Pseudomonas putida</i>		
<i>P.putida</i>	wild type, Prot-	H.K.Mahanty Lab Collection
<i>Serratia entomophila</i>		
A ₁ MO ₂	Derivative of A1 wild type, Amp ^R , Chi+, path+	Upadhyay <i>et al.</i> 1999

Plasmids used:

pUTZ2	pUT containing mini-Tn5KmlacZ2	deLorenzo <i>et al.</i> 1990.
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Table 2.1. List of Strains and plasmids used in the experiments described in Chapter 2

2.2.2. Media, reagents and antibiotics.

All media and reagents used in this study were prepared as described in Appendix I and II. Concentrations of the antibiotics used to maintain the selective pressure on the strains or the strains containing plasmid or cosmid constructs are as mentioned in Appendix I, unless otherwise stated.

2.2.3. Basic bacteriological methods.

B. gladioli pv. *agaricicola*, *Pseudomonas* and *Serratia* strains were grown under aerobic conditions, in a 30°C incubator. To isolate individual colonies of *Burkholderia* and *Pseudomonas* strains, plates streaked with bacteria on the selective plates had to be incubated for 36-40hr, while for *Serratia*, 20hr incubation yielded single colonies. *E. coli* strains were incubated aerobically at 37°C for 18hr. Well shaken liquid culture of the different strains grown in their respective temperature (mentioned above) for 18hr till the culture reached a stationary phase, were used as ‘overnight culture’ unless otherwise stated. For everyday use, plates containing strains were stored at 4°C and re-used for seven days. For long term storage, freezer stocks of strains and the constructs were made by growing bacteria overnight in LB supplemented with appropriate antibiotics, 1.5mL cultures were harvested by centrifugation at 7,000rpm for 2min at 4°C, resuspended in 800µL of fresh LB supplemented with 15% glycerol and stored at – 80°C.

Disc diffusion assay to test the antibiotic susceptibility of BG164.

BG164 was isolated from rotten mushrooms and purified cultures were stored in the culture collection of School of Biological Sciences at the University of Canterbury, Christchurch, New Zealand. No previous work on this strain included a description of genetic markers, such as antibiotic resistance.

In the assay, 50µL overnight culture of the test strain was aseptically spread on an LB plate, dried briefly in a sterile biohazard cabinet and impregnated with sterile antibiotic discs containing the concentration of antibiotics to be tested. The plates were incubated at 30°C for 24hr before scoring the susceptibility of the strains for the respective antibiotics. The assay was repeated at least twice in all cases.

2.2.4. Growth analysis of the strains.

For all the growth analysis experiments, 1:100 dilutions of overnight cultures of the test strain were grown in LB supplemented with or without antibiotics, as required for the specific experimental set up, incubated in shaking water baths, as mentioned in section 2.2.3. At regular intervals, 500µL aliquots of cultures were aseptically removed in replicates from the growing culture and the optical density of the culture at the specific time was measured at 260nm with a LKB Ultraspec[®] Plus spectrophotometer. When the viable cell counts at the various stages of growth were monitored, 500µL aliquot was removed and used as the stock sample to make serial dilutions. For enumerating the viable cells present in the individual dilutions, three aliquots of 5µL from each dilution were spotted on selective plates and incubated for 30hr at 30°C in case of all *Burkholderia* and *Pseudomonas* samples, unless otherwise stated.

2.2.5. Mushroom bioassay system.

The bioassay system established was an amendment of the basic technique implemented by Doreen G. Gandy in 1968, in which he had used blocks of mushroom tissue on wet paper towels maintained at 25°C in a Petri dish (Gandy, 1968). In routine assays, fresh

mushrooms supplied by a specific mushroom farm in Christchurch were washed thrice in sterile distilled water, cut into slices of approximately 1.5 to 2mm in width, with a sterile cut throat razor, and immersed immediately in ice cold water to prevent tissue browning. These pieces of mushrooms were aseptically transferred to sterile lunch boxes lined with wet paper towels, sterilized under a UV lamp for 15min and inoculated with the test bacteria, either as fixed volumes of sterile suspensions or as single colonies. The inoculated mushrooms were incubated for 16hr at 30°C. Within the time frame, the wild type bacteria caused distinct holes of approximately 4-5mm diameter on the mushroom pieces.

When growth of bacteria was monitored on mushroom slices, the mushrooms were cut into small pieces of approximately 0.5cm x 1.0cm and about 4mm thick, inoculated with the test bacteria. At regular intervals the pieces of mushrooms were taken out from the experimental set up with sterile forceps, vortexed for 2min in a sterile Eppendorf tube with 100µL of 1 x MS. The supernatant was used to make serial dilutions and three aliquots of 5µL from each dilution were spotted on selective plates and incubated for 30hr at 30°C.

2.2.6. Mutagenesis.

Mini-Tn5KmlacZ2 (deLorenzo *et al.*, 1990) has been used to generate random insertional mutants, primarily because of the kanamycin resistance marker that could be easily traced in BG164R apart from some other salient features. This Tn5 derivative is capable of fusing *lacZ* to whatever gene it disrupts. The *lacZ* gene lacks both transcriptional and translational signals, and the *lacZ* fusion protein created in each case of successful mini-Tn5KmlacZ2 transposition in the right reading frame of a gene is separated from the target gene by 49 nucleotides. Additionally, the transposon has a functional kanamycin gene, which facilitates the one step selection of the mutants *in vitro*. The transposon has been successfully used in creating random protein fusions in *P. putida* (deLorenzo *et al.*, 1990), and in some other enteric and soil borne Gram-negative bacteria (Monds *et al.*, 2001; Giddens *et al.*, 2002).

The delivery vehicle used for the transposon mini-Tn5KmlacZ2 is pUTZ2, which has been constructed in the plasmid vector pUT (Herrero *et al.*, 1990), a pGP704 derivative. pUT plasmid has the π protein dependent RK6 origin of replication. As a result, it serves as a suicide vector when conjugated into strains that lack chromosomally borne π protein. It also has an RP4 origin of transfer (*oriT*) and so it can be mobilised only when conjugated from an *E. coli* (Eg: S17-1 λ pir) strain that supplies the RP4 conjugative functions in trans. The transposase gene required for transposition of mini-Tn5 is also supplied in *trans* by the vector. Once integrated into the target gene, the transposon is stably maintained without any further transposition event.

2.2.6a. Conjugation protocol used for the generation of mutants.

The transposon mini-Tn5KmlacZ2 borne by pUTZ2, maintained in *E. coli* S17-1 (λ -pir) was introduced into the recipient, BG164R, by conjugation. Overnight cultures of the donor *E. coli* S17-1 (λ -pir) (Simon *et al.*, 1983) containing pUTZ2, and the recipient strain, *B. gladioli* pv.*agaricicola* BG164R were washed with fresh LB broth to remove the respective antibiotics from the media, the recipient was heat shocked at 43°C for 15min and mixed with the donor in a ratio of 4:1. The mixture was spotted on LB plates without any antibiotics and incubated at 30°C for 4hr, after which the cells were recovered from plates by washing with fresh LB broth and centrifuged. The resulting pellet was resuspended in 200 μ L of fresh LB broth and 100 μ L aliquots were spread on selection plates consisting of rifampicin, kanamycin and chloramphenicol (15 μ g/mL) to select for BG164R transconjugants. It was observed that the transconjugants started appearing on the plates after about 30hr of incubation, but to get colonies of reasonable size, which would be easy to screen on mushroom assays, the plates had to be incubated for at least 36hr.

2.2.6b. Screening and isolation of mutants with ‘no-cavity’ phenotype.

Transconjugants arising from independent conjugations were screened for the transposition events of the miniTn-5 resulting in ‘no-cavity’ phenotypes using the mushroom assay described in section 2.2.5, as well as on minimal agar plates to test the

number of auxotrophic mutants to test the randomness of the transposition events of the transposon in the chromosome.

2.2.7. Phenotypic characterisation of mutants.

2.2.7a. Preparation of samples for Transmission Electron Microscopy (TEM).

TEM grids were prepared by scraping 24hr old bacterial cells from plates supplemented with appropriate antibiotics, re-suspended in 100 μ L of sterile distilled water and two drops of this 1% phosphotungstic acid, and spotting onto TEM grids. Extra solution was drained off by capillary action from the grids by using a filter paper. Stained bacteria were observed under bright field either in a Hitachi H-600 Electron microscope in the Mechanical Engineering Department or with a JEOL JEM-1200EX Electron microscope available in the School of Biological Sciences, at the University of Canterbury.

2.2.7b. Motility Assay.

The capacity of movement in the different species of bacteria is generally tested by spotting test bacterium on plates supplemented with low percentage of agar. Motility agar plates supplemented with 0.3% agar as stated in Appendix I were used for the assay. Motility in such cases was defined by the capacity of the test bacteria to move away from the point of inoculation on the plate, thereby forming a colony, which appears more like a halo around the central inoculation point. Strains were assessed non-motile when there was no halo formation around the central inoculation site.

2.2.7c. Biofilm Assay.

The assay conditions standardised after assessing the biofilm forming capacity of wild type strain in different media and with varying hours of incubation were: 16hr incubation of 1:50 dilution of stationary cultures of wild type and mutant cells at 30°C in 100 μ L of mushroom extract media (Appendix I) in 96 well microtitre dishes or 1mL of media in borosilicate glass test tubes. Biofilms were visualised after staining with crystal violet for 20min at room temperature, followed by rigorous washing under flowing water and drying by tapping on paper towels.

2.2.7d. Scanning Electron Microscopy (SEM).

Mushroom slices were inoculated with the test bacteria growing on LB plates with respective antibiotics and incubated for 16hr as per the established mushroom bioassay conditions. After the required incubation times, the mushroom slices were washed with phosphate buffer (Appendix II) to remove excess loosely bound bacterial cells, frozen in liquid nitrogen, sprayed with gold dust and observed under Leica s440 SEM, available in the department.

2.2.7e. Chitinase Assay.

Chitin plates prepared according to the method stated in Appendix I were used in the assay procedure. The test bacteria were spotted on the plates and incubated at 30°C for 4days before scoring the ability of the bacteria to produce chitinase. Production of chitinase was determined by the clearing of colloidal chitin around the colonies spotted on the plates.

2.2.7f. Protease Assay.

The ability of test bacteria to produce protease was tested by transferring the colonies onto 1% skim milk agar plates (Appendix I) and checking the capacity of the strains to clear casein around the test colonies. The plates were incubated at 30°C for 24hr before noting the ability of the test bacteria to degrade casein.

2.2.7g. o-DPO inhibitions Assay.

The o-DPO assay was adopted from the method standardised by Ferrar in 1995 (Ferrar, 1995), whereby commercially available mushroom o-DPO was used as the enzyme, and was allowed to react with a substrate DOPA (dihydroxyphenylalanine). The reaction resulted in the formation of a coloured product DOPA-chrome and could be measured at OD 452 nm. The intensity of the colour produced was taken as a measure of the amount of active tyrosinase present in the reaction. The culture extract from the potential microorganisms were used to test the presence of potential inhibitory compounds. In the assay, 50µL of the enzyme (0.252AU at 452nm) was added to 100µL 0.2M phosphate buffer pH7.0 and 50µL of 10mM DOPA in a 96 well microtitre dish. To this, 100µL of

the test supernatant was added and incubated for 20min at 30°C. After the required incubation time the OD at 452nm was read on a multiscan reader available in the department.

2.2.7h. In vitro antifungal phenotype.

The method used was an adaptation of the method stated by Carruthers *et al.* 1994 (Carruthers, 1994). Plugs of agar containing actively growing fungal mycelia were inoculated at the centre of PDA plates (Appendix I) and incubated at 23°C for 2 days until the radius of the freshly subcultured test fungus was about 3.0cm. Colonies of test bacteria were streaked with a sterile loop approximately 1cm away from the growing mycelia tip and the plates were further incubated for 2 days before checking the extent of inhibition in comparison to the growth of fungal mycelia on a plate which did not have any bacteria growing on it.

2.3. Results.

2.3.1. Describing the genotype of BG164

The antibiotic resistance profile of BG164 was determined because the genotype of the strain was previously unknown and was essential for gene manipulation experiments. BG164 was susceptible to a number of commonly used antibiotics as determined by disc diffusion assay technique, (Table 2.2). To select the strain in subsequent analyses, a rifampicin resistant derivative was isolated.

Susceptible to:	Resistant to:
Ampicillin 100µg/mL	Chloramphenicol 50µg/mL
Nalidixic acid 15µg/mL	Polymyxin 300µg/mL
Tetracycline 30µg/mL	Erythromycin 15µg/mL
Rifampicin 50µg/mL	Vancomycin 30µg/mL
Kanamycin 50µg/mL	Penicillin 10µg/mL
Gentamycin 50µg/mL	Streptomycin 50µg/mL

Table 2.2: Antibiotic susceptibility of BG164.

Spontaneously arising rifampicin-resistant BG164 colonies were isolated by plating 100µL of 1: 100µL of overnight culture of BG164 cells containing approximately 10⁶ cells, on LB plates supplemented with rifampicin (50µg/mL) and incubating them at 30°C for 48hr. Eight spontaneous rifampicin-resistant colonies initially appeared on the selection plates. All the colonies were further tested by re-streaking on to LB plates supplemented with 50µg/mL Rifampicin. Four representative colonies out of the 8 spontaneous mutants were grown in liquid cultures to test the capacity of growth in antibiotic supplemented liquid media. After 18hrs of growth, 3 out of the 4 colonies tested, grew to a uniform Optical Density (OD) at 260nm wavelength (OD₂₆₀= 1.7). To further test the stability of the marker in the strains, two representative colonies growing in rifampicin supplemented LB broth were subcultured (1:100 dilution) in LB broth without any antibiotics, serially diluted and all dilutions plated in triplicate on LB plates with and without antibiotics. Colonies arising on the plates were enumerated for comparison (Table 2.3).

Strain	CFU/mL	
	On LB plate	On LB +Rif plate
BG164-3	8.2X10 ⁹	1.1X10 ¹⁰
BG164-5	7.15X10 ⁹	9.1X10 ⁹

Table 2.3: Stability of the Rifampicin resistant BG164 strains created.

There was no major difference (Table 2.3) in the number of colonies appearing on either of the plate types indicating that the spontaneous mutation was stable. Eventually, BG164-5 was designated as **BG164R**, and used in all the experiments described henceforth.

2.3.2. Introduction of Rifampicin resistance in BG164R does not affect virulence.

The virulence of BG164 and BG164R was compared by monitoring Cavity disease symptoms on mushrooms in two parallel experiments. In the first experiment, overnight cultures of BG164 and BG164R were re-suspended in sterile distilled water to a concentration of 1×10^9 cells/mL. Aliquots of 2 μ L and 4 μ L were randomly spotted onto mushroom caps, which were then incubated for 20hr at 30°C. In a second setup, colonies were transferred by toothpicks onto the surface of mushroom caps and incubated at 30°C for 20hr. No difference was observed between the strains in the development of symptoms by either set-up (Figure 2.1). BG164R was thereafter selected for use as the test strain for further studies.

The primary objective was to identify the genetic mechanism(s) by which *B. gladioli* pv. *agaricicola* causes cavity disease in mushrooms. The strategy adopted to identify the genes involved was random mutagenesis screens, which were followed by the isolation of mutants unable to form cavities on mushrooms. In order to trace such phenotypes in

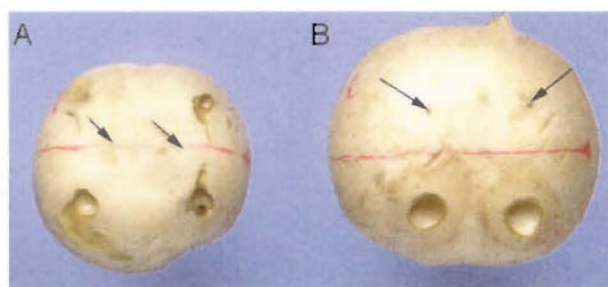


Figure 2.1A: Inocula consisting of liquid suspensions in water. Top left 2 μ L of BG164 inocula. Top right 2 μ L of BG164R. Bottom left 4 μ L BG164 suspension and bottom right 4 μ L of BG164R. **In 2.1B:** Inocula consisting of toothpicked colonies; left BG164 colony and right BG164R colony. Arrows indicate sites of control inoculations with water (A) and blank toothpick marks (B).

vivo, a large number of random mutants would need to be screened, for which the establishment of an efficient bioassay system with conditions best suited for proper representation of the disease was essential.

2.3.3. Establishment of bioassay system to study Cavity Disease.

Initially, whole mushroom sporocarps without the stock were surface sterilised, placed in a moist chamber and inoculated with *B. gladioli* pv.*agaricicola* to test for pathogenicity. However, to screen large numbers of mutants, an assay using whole sporocarps was not practical. Therefore, a wide range of incubation times and mushroom pieces (Figure 2.2.) of different sizes were tested to best represent Cavity disease and to serve our purpose of screening maximum number of mutants per set up.

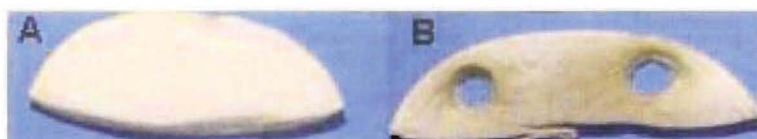


Figure 2.2: A representative mushroom bioassay used for the screening of mutant bacteria. [A] Control Slice. [B] Mushroom slice with cavity formed by BG164R under the defined assay conditions.

For screening of mutants, mushroom pieces were lined in 4 columns of 7 rows in each lunch box. On each piece of mushroom four colonies of test bacteria could be patched. A total of 100 colonies could be tested for the desired phenotypes, in each lunch box used, with the appropriate negative and positive controls.

2.3.4. Establishment of the mutagenesis system.

Mutations in the virulence genes of BG164R were created by mini-Tn5KmlacZ2 mutagenesis. In an effort to get the maximum number of transconjugants, two parameters had to be optimised. Firstly, the time of incubation of conjugants and secondly, the ratio of donor to recipients to yield maximum number of transformants for a given incubation time.

In a series of preliminary experiments, the two variables were measured by mixing conjugants in different proportions and incubating mixtures for various times. To establish optimum ratio of donor to recipients, respective strains were mixed in ratios of 1:1, 1:2, 1:3 and 1:4; and, to establish the best suited incubation period, 1hr, 2hr, 4hr and 6hr were tested. Cells were recovered from the test plates by washing with fresh LB broth, centrifuged and the pellet was plated on selection plates. Transconjugants started appearing on the plates after about 30hr of incubation. Incubation periods of 36hr were necessary to get colonies large enough for further work. However, by that time spontaneous rifampicin-resistant donors also began to arise on the control plates, making it difficult to select true transconjugants from the conjugation plates. To circumvent this problem, the selection plates were supplemented with chloramphenicol (15mg/mL). The optimal conditions established for the experiment were 250 μ L of overnight culture of donor to be mixed with 1mL of heat shocked overnight culture of recipient and conjugated for 4hr at 30°C. The transconjugants were selected on rifampicin, kanamycin and chloramphenicol supplemented LB plates and incubated for a further 36hr before screening for mutants.

2.3.5. Selection of 'no-cavity' mutants.

Approximately 10,000 transconjugants arising from 66 independent experiments carried out in 16 attempts were screened for the transposition of the miniTn-5, resulting in 'no-cavity' phenotypes using the mushroom assay described in section 2.2.5, as well as on minimal agar plates to test the number of auxotrophic mutants. On average, 1.5% of the transconjugants were auxotrophs, thereby suggesting that the transposition events in the chromosome were randomly distributed.

Twenty 'no-cavity' forming mutants were selected after testing each putative BG164R mutant three times on mushroom slices. Of these 20 mutants, 6 were prototrophic (Figure 2.3.) and the remaining 14 were unable to grow on minimal media. The genotypes of the 6 prototrophic mutants isolated have been described in Table 1.1.

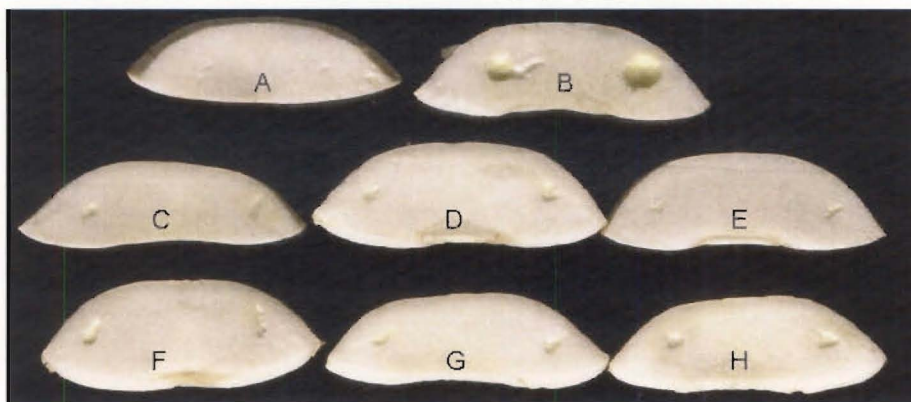


Figure 2.3: Mushroom Bioassay to compare between the wild type, BG164R and the mutants selected for no cavity phenotype. [A] Control prick, [B] BG164R, [C] BG4-12, [D] BG12-88, [E] BG12-147, [F] BG15-40, [G] BG15-87 and [H] BG16-787

Was the 'no-cavity' phenotype, evident in the mutants isolated, due to the inability of the mutant strains to grow on the mushroom slices? In the formation of Cavity disease, BG164R has to establish itself amongst endogenous mushroom bacteria before causing disease, as will be described in chapter 5. If mutants have growth defects, they might be less able to compete with the endogenous bacteria (refer section 5.3.4). This could lead to the no-cavity phenotype and not be an effect of a mutation in a virulence gene. Thus, to test the ability of mutants to grow on the mushroom slices, growth rates of the mutants were compared to that of the wild type.

2.3.6. Comparison of the rate of growth of mutants with the wild type.

The growth rate of the wild type and the mutants were monitored over 12hr in LB broth supplemented with required antibiotics, by measuring the optical density of the cells at 600nm at a regular interval (section 2.2.4). The viable counts of the bacteria were also monitored over time, by serially diluting the cultures and plating them on the respective selection plates. Data arising from triplicate sets of independent readings were used to draw the growth curves and calculate standard errors.

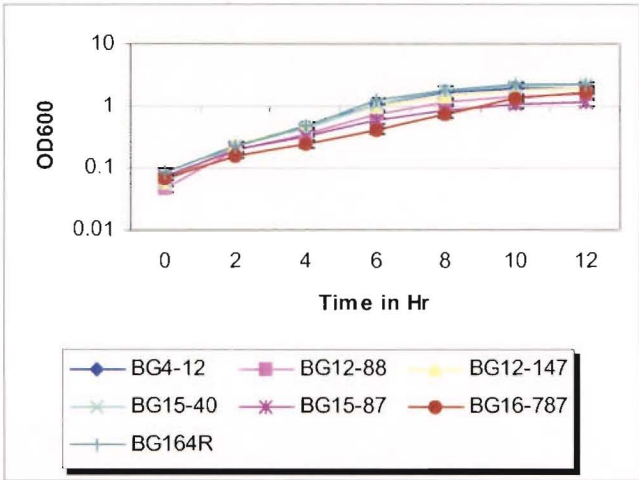


Figure 2.4.A: Comparative growth (OD₆₀₀ values) of the mutants with the wild type strain, BG164R, in LB broth.

The mutants and the wild type grow at nearly the same rates (Figure 2.4.A). At the end of 12hr, most of the strains grew up to an OD₆₀₀ of 2.0. Among the different mutants, the patterns of growth observed in the case of BG4-12, BG15-40 and BG12-147, were similar to that of the wild type, BG164R. The other three seemed to be growing slower, with BG15-87 being the slowest. BG15-87 grew only to an OD₆₀₀ of 1.2. Evidently, this strain also shows a gradual decline in the CFU/mL (Figure 2.4B.).

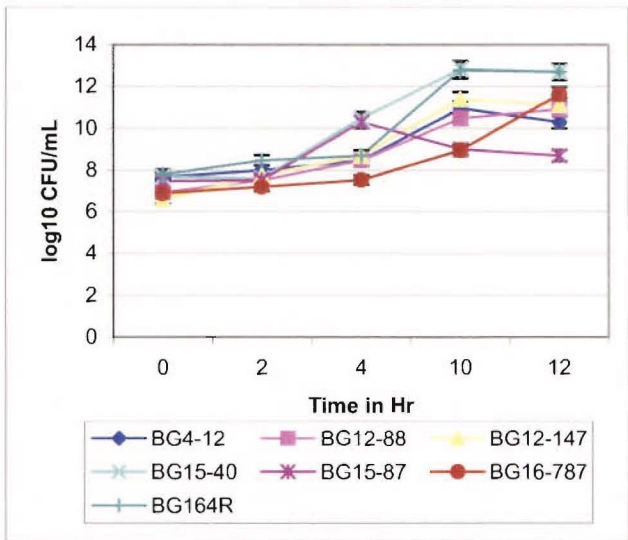


Figure 2.4.B: Comparative growth (viable cell counts) of the mutants and the wild type strain, BG164R in LB broth.

This could be attributed to the initial difference in the viable cell counts at the start of the experiment (Figure 2.4.B), which shows the trend of growth calculated as viable cell counts over a 12hr period. The growth rates, as evident from viable cell counts are very distinct for each strain tested. This could partially be due to sampling errors or genuinely due to the effects of mutation. It was difficult to conclude at that stage without having an idea of the genes that have been disrupted in each case.

In order to monitor the rate of growth of the mutants on mushroom slices, two separate experiments were set up. The growth of a representative auxotrophic and a representative prototrophic mutant in comparison to that of the wild type strain was monitored over a 16hr period. In both the experiments, 10 μ L aliquots of 1:100 dilution of an 18hr culture of the wild type, and the two different mutants were spotted onto mushroom slices and incubated for 16hr at 25°C. At regular intervals, the bacteria were retrieved from the mushroom slices by agitating the slice in 100 μ L of LB for 30sec in a vortex mixer, serially diluted and plated on selection plates. The data presented in Figures 2.5.A and B are the average number of cells retrieved from slices taken from three separate samples. The auxotrophic mutant grows in the initial 6hr, but thereafter does not show any increase in CFU/mL over a period of 16hr on the mushroom slices (Figure 2.5.A). In fact, there was a very slow decline in the population of the auxotrophic mutants compared to wild type observed over time in the mushroom background, which shows a clear trend of increase in the population with time. In contrast, the prototrophic mutants showed clear evidence of gradual increase in number of cells, which was comparable to that evident in the case of BG164R. In the figures, a difference in the number of cells is observed in case of the initial inoculum and the number of cells retrieved after 4hr incubation on the mushroom slices. This was because of the technical problems associated with retrieving live cells from the mushroom tissues after inoculation. This problem has been consistently observed in case of all experiments, which involved extraction of live cells from mushroom tissues. The proportion of cells that could be extracted from mushroom slices immediately after inoculation was estimated. Approximately 25-30% of the population was lost in each case.

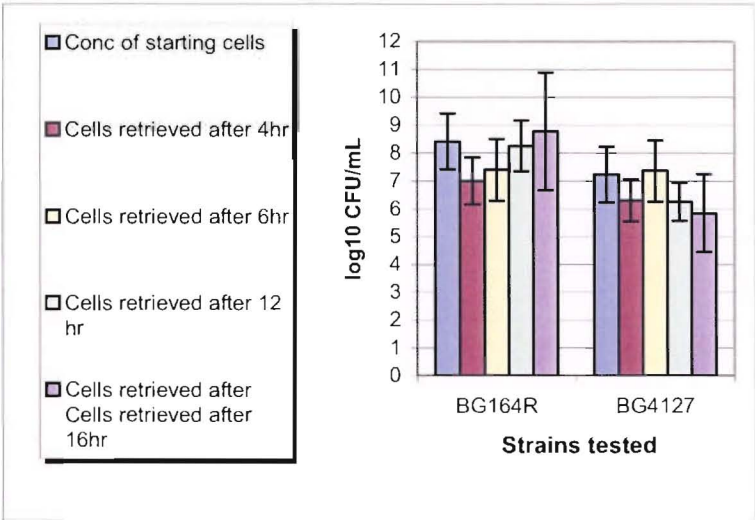


Figure 2.5.A: Comparative growth rates of BG164R against the auxotrophic mutant BG4-127 on mushroom slices.

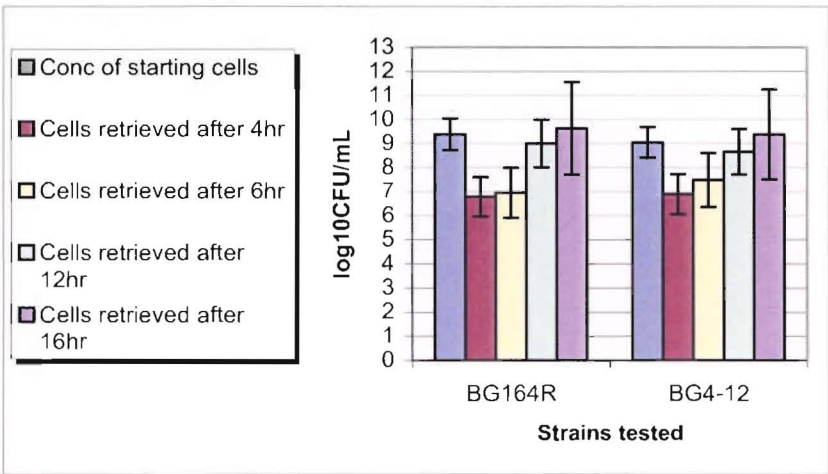


Figure 2.5.B: Comparative growth rates of BG164R against the prototrophic mutant BG4-12 on mushroom slices.

The ‘no-cavity’ phenotype observed in the auxotrophic mutants could thus be due to the mutation itself or due to the inability of the strain to grow on mushrooms. Analysis of the auxotrophic mutants in relation to no-cavity phenotype would have been interesting, but, since the main objective of the research was to identify the genes that directly had a role in the formation of cavity disease, it was decided to focus on and analyse the prototrophic mutants.

The ability of a pathogenic bacterium to cause disease has been well documented to be associated with certain macromolecular bacterial surface components like flagella (Tomich *et al.*, 2002; Chua *et al.*, 2003), pili (O'Toole & Kolter, 1998b; Pratt & Kolter, 1998; O'Toole *et al.*, 2000), and capsular polysaccharides (DeShazer *et al.*, 2001). These structures can be observed easily under the microscope or analysed phenotypically by simple assays. These techniques were therefore used to look for obvious changes in the mutants in comparison to the wild type.

2.3.7. Transmission Electron Microscopy.

To find out whether the mutations had any effect on the morphology of the cell surface, transmission electron microscopy was used to observe the physical appearance of the mutants as compared to that of the wild type strain. Entire surfaces of the grids containing samples prepared according to the method mentioned in 2.2.7a were scanned under different magnifications and representative pictures of the entire field are presented in Figure 2.6.

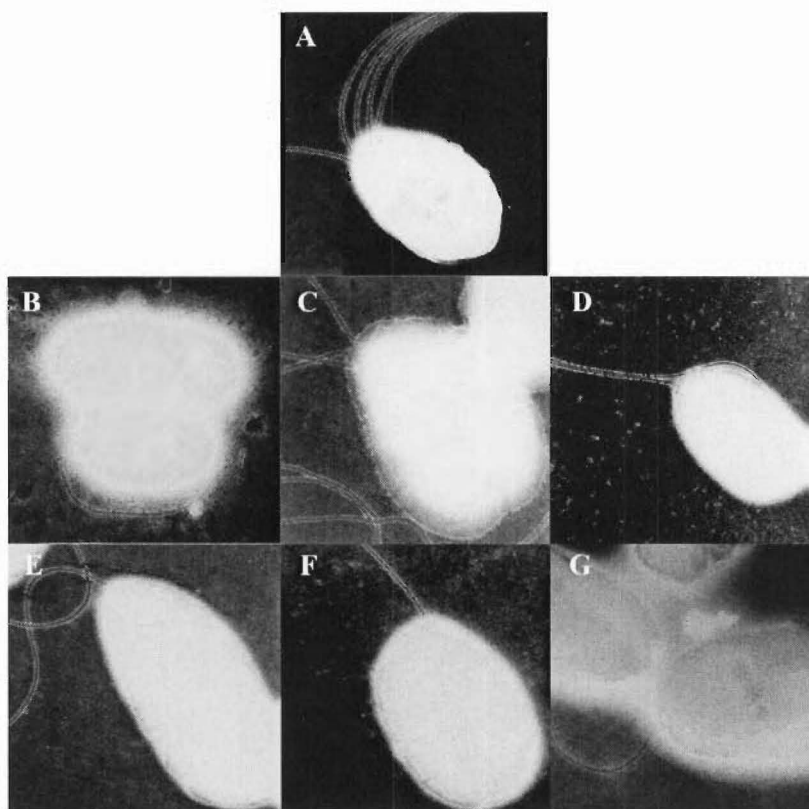


Figure 2.6: Transmission electron micrographs of the mutants and the wild type cells. [A] BG164R, [B] BG4-12, [C] BG12-88, [D] BG12-147, [E] BG15-40, [F] BG15-87, [G] BG16-787. Magnification is = X30, 000, except C and G, where the magnification is = X60, 000.

The wild type strain has 5 polar flagella. Four out of the six mutants have a single polar flagellum, while two strains BG12-88 and BG12-147 had two visible polar flagella. Apart from the changes in flagella number, no major change in the morphology on the cell surface was observed.

Flagella are organelles required for motility and chemotaxis of the cell (Macnab, 1992), however, having them has also been correlated with the ability to form biofilms (O'Toole & Kolter, 1998b; Pratt & Kolter, 1998; Watnick & Kolter, 1999) and for virulence (Lillehoj *et al.*, 2002; Tomich *et al.*, 2002; Chua *et al.*, 2003). Flagella-mediated motility has been associated with pathogenicity in *P. aeruginosa* (Lillehoj *et al.*, 2002), *Campylobacter jejuni*, *Helicobacter pylori*, *Vibrio cholerae* (Dasgupta *et al.*, 2000), *B. pseudomallei* (Chua *et al.*, 2003), *Proteus mirabilis*, *C. jejuni* and *B. cepacia* (Tomich *et al.*, 2002). Studies on the mechanisms by which flagella effect virulence and pathogenicity of the different bacterial species have been made by comparing either aflagellate, non-motile mutants, or mutants that over-produce flagella. In the case of Cavity disease, the 'no-cavity' mutants of BG164R had a reduction in the number of flagella. So, the probability of alteration in the motility of BG164R due to reduction in the flagella number was addressed, as, differences in motility could ultimately affect the capacity of disease formation.

2.3.8. Motility Assay.

Motility in strains belonging to the family Pseudomonaceae have generally been tested in plates made with 0.25% to 0.3% agar (Dasgupta *et al.*, 2000; Monds, 2000; Tomich *et al.*, 2002; Chua *et al.*, 2003). Non-motile mutants of *Burkholderia* sp., have been tested on 0.25% and 0.3% agar and clear differences in the phenotype could be observed. On the basis of these published data, 'no cavity' mutants of BG164R, with reduced numbers of flagella, were initially tested by transferring individual colonies of the different mutants on motility agar plates (section 2.2.7b.). No difference in the colony morphology was observed in any of the mutants when compared to the wild type strains, although, clear differences were observed in the control PA147-2 (Carruthers, 1994) wild type motile and the control non-motile mutant strain, PAE639 (Monds, 2000). The motility of BG164R on 0.3% agar was prolific and within 18hr of incubation the cells spread widely on the agar plates, making big diffused colonies. Thereafter,

plates with varying proportions of agar ranging from 0.2% to 0.8% were tested to check for any visible differences in the motility patterns of wild type and mutants. In none of these cases changes in the capacity of swimming of the mutants were observed, when compared to the wild type.

Flagella mediated motility have been co-related to the formation of bacterial biofilms in *E. coli*, *P. aeruginosa* and *P. aureofaciens* (O'Toole & Kolter, 1998b; Pratt & Kolter, 1998; Monds *et al.*, 2001). Most of these reports are based on results of studying aflagellate mutants of the wild type strain. Whether reduction in the number of flagella, as observed in case of the 'no-cavity' mutants of BG164R, had any effect on its capacity to form bacterial biofilms was thus an interesting feature to trace in the mutants

2.3.9. Biofilm Assay.

Bacterial biofilms are defined as "matrix enclosed bacterial populations adherent to each other and /or to surfaces" (Pratt & Kolter, 1998). Pathogenic bacteria form and live within such sessile communities on the surface of living tissues. Apart from possessing flagella, a number of other features (O'Toole & Kolter, 1998b; Pratt & Kolter, 1998; Monds *et al.*, 2001), like the formation of type I and type IV pili, production of exopolysaccharides and certain environmental cues based mainly on the availability of nutrients (O'Toole *et al.*, 2000) have been found to be associated with the formation of bacterial biofilms.

Although under natural conditions bacterial biofilms develop on biotic surfaces, laboratory based research on biofilm studies are generally done by growing bacterial cells on different hydrophobic abiotic surfaces, such as plastics made of polyvinyl chloride (PVC), polycarbonate and polypropylene or on hydrophilic surfaces as borosilicate glass (O'Toole & Kolter, 1998a). Laboratory studies have demonstrated that although the extent of biofilm formation by the wild type *P. fluorescens* strain WCS365 varies on different hydrophobic surfaces, a comparative study based on biofilm formation by the wild type and no biofilm forming mutants yield similar results in all the model hydrophobic surfaces tested (O'Toole & Kolter, 1998a). However, in such comparative assays with wild type strains and mutants, differences could be observed on the capacity of biofilm formation on hydrophobic and hydrophilic surfaces.

The main purpose of studying biofilm forming capacity in case of the Cavity disease pathogen was to find out any possible observable differences in the capacity of biofilm formation by the non-cavity forming mutants in comparison to that of the wild type. Thus, although the study was carried out using abiotic surfaces, in an effort to imitate the nutritional conditions present in mushroom, biofilm studies on the mutants and wild type was done with mushroom juice extract media (Appendix I). Initially, a whole range of media and two types of abiotic surfaces, hydrophobic PVC and borosilicate glass were tested, and the assay conducted according to the conditions stated in section 2.2.7c.

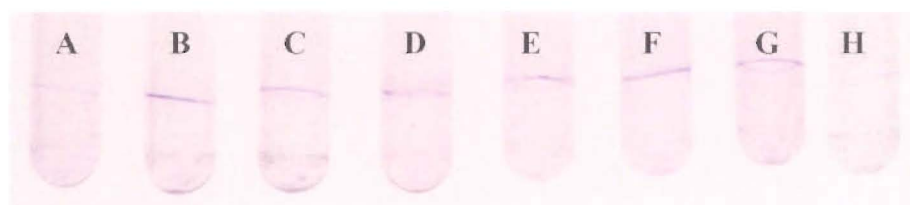


Figure.2.7: Biofilm formation by wild type BG164R and its Cavity minus mutants. [A] BG164R, [B] BG4-12, [C] BG12-88, [D] BG12-147, [E] BG15-40, [F] BG15-87, [G] BG16-787, [H] Media control.

On PVC plastic hydrophobic surfaces, the biofilm formation was weak and no differences could be noticed when the mutants were compared. Of the different media tested and the surfaces tested, best biofilm formations were observed in borosilicate glass test tubes and in mushroom juice extract media (Figure 2.7.). There was no distinct difference in the intensity of crystal violet staining of the biofilms, as a measure of biofilm formation capacity, formed by the mutants in comparison to the wild type on the hydrophilic surface.

2.3.10. Scanning Electron Microscopy.

Electron microscopy was used to visualise the effect of growth of the wild type and mutant bacteria on the morphology of mushroom tissues. Mushroom slices were inoculated with bacteria and incubated for 16hr prior to preparing samples for the microscope (2.2.7d).

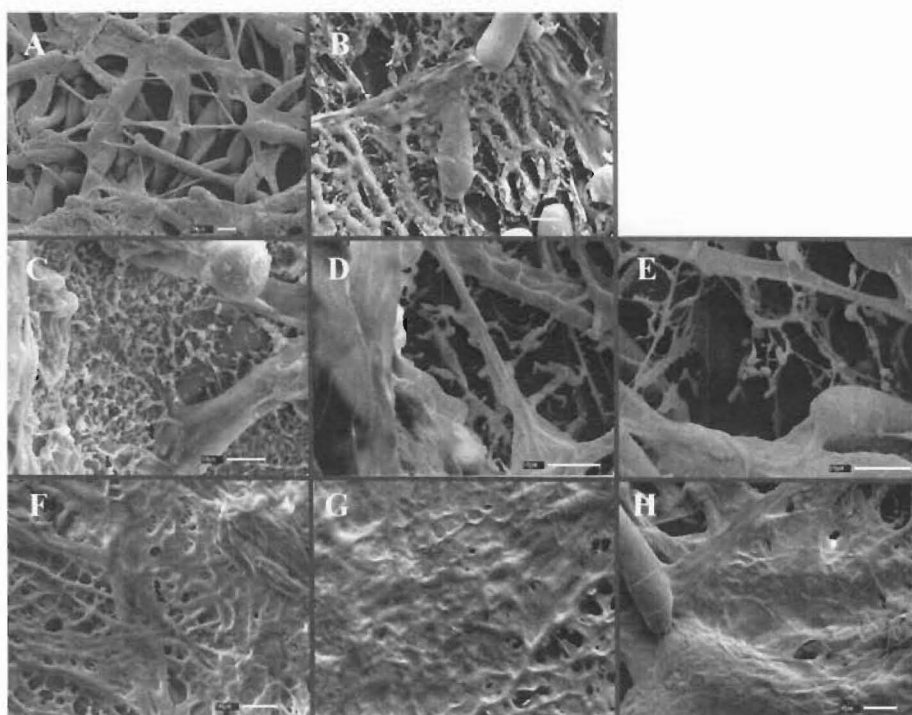


Figure 2.8: SEM of mushroom tissues inoculated with the wild type and mutant bacteria. [A] Control mushroom slice without any bacteria showing intact mushroom mycelia, [B] Degraded mycelia on being challenged with the wild type strain BG164R, [C-H] Mushroom slices challenged with mutants. [C] BG4-12, [D] BG12-40, [E] BG12-88, [F] BG15-40, [G] BG15-88, [H] BG16-787

In general, the mutants (Figure 2.8.C-H) formed a continuous sheath on the surface of the mushroom pieces, with occasional breaks under which the mushroom hyphae were visible. In comparison, mushroom slices inoculated with the wild type bacteria remained attached to (Figure 2.8.B) skeletal remains of degraded hyphal filaments. Mycelial filaments associated with the mutants were shrivelled under the bacterial sheaths (Figure 2.8F-H). Filamentous strands of bacterial cells appeared in the breaks of the bacterial sheath (Figure 2.8.C-E), although they cannot be associated with the degraded masses of hyphae as observed in case of the wild type strains. Thus, under the standardised assay conditions, it was concluded that mutants fail to degrade the hyphae of mushrooms, but, were able to shrivel mushroom mycelia. The wild type strain, however, clearly degraded mushroom tissue and, to degrade fungal cells, bacteria would have to secrete certain degradative enzymes.

The production of enzymes that degrade mushroom tissue could be the explanation for the effect of the wild-type strain on the mushroom tissue. BG164R has been associated

with the production of both chitinase and β -glucanase, as well as other bioactive compounds, essentially toxins (Gill & Tsuneda, 1997). In combination, chitinase and glucanase act as powerful degraders of mushroom tissue, but if present singly, tissue degradation is not as extensive (Gill & Tsuneda, 1997). Gill and Tsuneda (1997) proposed the necessity of combined action of both the enzymes since, chitin and β -glucan are inextricably covalently linked in case of fungal cell walls {Sietsma & Wessels 1979, cited by Gill & Tsuneda 1997}, with chitin forming the inner skeleton and β -glucans forming the matrix. Thus, in order to degrade fungal mycelia, depolymerisation of chitin would be a key component. Although Gill and Tsuneda (1997) reported the production of chitinase by the strain, other researchers working on this strain (Young, 1992) could not observe chitinase activity when pure colonies growing under laboratory conditions were tested.

2.3.11. Chitinase Assay.

The capacity of BG164R and the mutants to degrade colloidal chitin were tested using the plate assay technique stated by Gill & Tsuneda (1997) and described in section 2.2.7e. In comparison to the wild type (Figure 2.9), the mutants had a highly reduced capacity to degrade colloidal chitin. The effectiveness of the assay was confirmed using *Serratia entomophila* strain A₁MO₂ as positive control and *P. cepacia* strain B111 as a negative control.

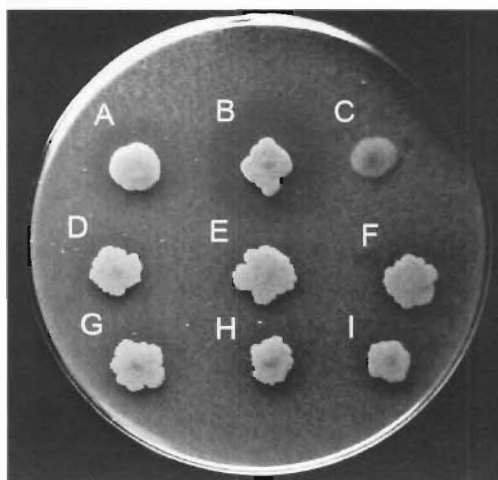


Figure 2.9: Chitinase assay. [A] *Serratia entomophila* strain A₁MO₂ Chi+(Positive control) [B] BG164R [C] *P.cepacia* strain B111 (negative control) [D] BG4-12, [E] BG12-88, [F] BG12-147, [G] BG15-40, [H] BG15-87, [I] BG16-787.

Soft rot causing bacteria in higher plants have been associated with the production of proteinases apart from pectinases and cellulase. A mushroom soft rot pathogen was thus quite naturally expected to produce chitinase; that would degrade the structural component of mushroom tissue. It is evident that a chitin degrading enzyme is produced by the strain. The other common group of enzyme produced by soft rot pathogens is protease and so, the ability of mutants to degrade milk protein was tested thereafter.

2.3.12. Protease Assay.

Protease production was tested using a standard *in vitro* assay (2.27f). Colonies of the wild type and the mutant strains were transferred onto 1% skim milk agar plates and incubated for 24hr at 30°C. No protease activity was detected by this assay using the mutant strains (2.10).

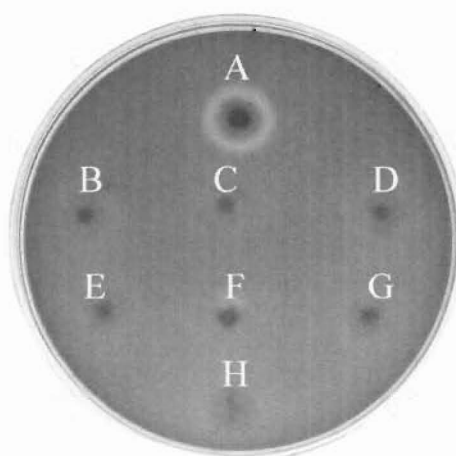


Figure 2.10: Protease assay on milk agar plates. [A] BG164R Wild type strain, [B] BG4-12, [C] BG12-88, [D] BG12-147, [E] BG15-40, [F] BG15-87, [G] BG16-787, [H] *P.putida* (-ve control).

Protease secretion, rather than production explains the protease minus phenotype in members of the closely related pathogenic *Burkholderia* sp. Generally, in the case of secretory mutants, protease secretion is also linked to the capacity to secrete a range of virulence factors. The mutants described here could, therefore, have insertions in genes for secretion. If they were secretory mutants, then it could be expected that the secretion of other virulence factors were also effected. The wild type strain is known to secrete an inhibitor of *ortho*-Diphenol oxidase (o-DPO), an enzyme associated with host defence (Aldridge & Walker, 1980). The next question that was addressed was whether the

Cavity disease mutants of BG164R lost the capacity of production or secretion of this inhibitor.

2.3.13. *o*-DPO inhibition assay.

The *o*-DPO inhibition assay (2.2.7g) was used to check any difference in the capacity of the various mutants to inhibit mushroom *o*-DPO. The positive control strain, BG164R, in contrast to that reported by Ferrar (1995) did not produce a detectable inhibitor of *o*-DPO over an incubation time of 20min. The assay was repeated with a reduced incubation time of 10min. Even with the reduced time of incubation, only 10% inhibition of tyrosinase was observed in comparison to 20% reported by Ferrar (1995). Despite these inconsistencies between the published observations and those reported here, the supernatants from the mutants were compared in the assay. There was also an inhibition of 10% enzyme activity, suggesting that the mutations did not affect the production or secretion of the tyrosinase inhibitor produced by the wild type strain. Variation in the data reported earlier and the data obtained could be attributed to the difference in the batches of the commercial enzyme used in the assay. Although, any difference in the secretion of tyrosinase inhibitor was not detected in the supernatants, secretion of other mycelia degrading compounds could have been affected by the mutations.

Gill and Tsuneda (1997) designated *B.gladioli* pv. *agaricicola*, BG164R as a 'novel mushroom pathogen' based on their finding that the cavity disease pathogen produces both toxins and degradative enzymes that degrade mushroom mycelia. They associated the production of toxins by the strain with the capacity to inhibit fungal mycelia. To test whether the mutants had any change in the ability to inhibit fungal mycelia, actively growing mycelia were challenged with the mutant bacteria.

2.3.14. *in vitro* antifungal phenotype.

An *in vitro* antifungal inhibition assay was set up to test whether the mutants lost the capacity to inhibit fungal mycelia. Initially, growing mycelia of *Gaeumanomyces graminis* were tested because BG164R was the strongest inhibitor (2.2.7h) of it.

Thereafter, the observation was extended to the capacity of inhibition of mushroom mycelia on CMA plates by two representative mutants. The no-cavity causing mutants were still able to inhibit the growing mushroom mycelia (Figure 2.11). Similar

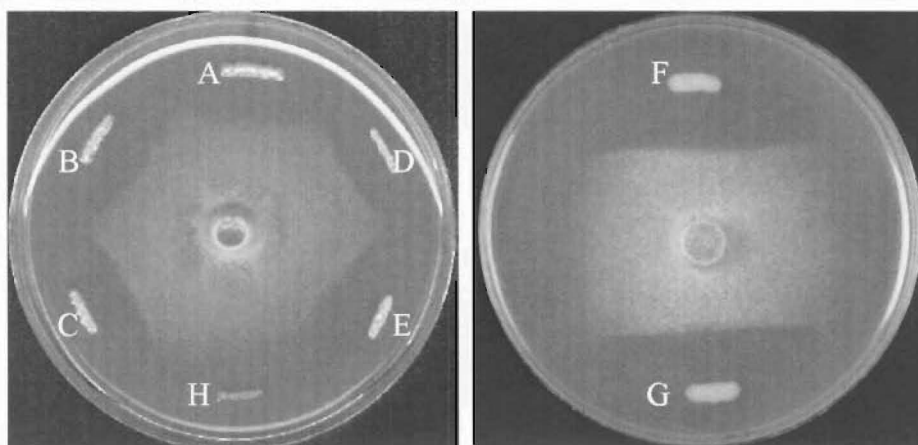


Figure.2.11: Inhibition of *Gaeumanomyces graminis* mycelia by wild type BG164R, and its 'no-cavity' causing mutants. [A] BG164R, [B] BG4-12, [C] BG12-88, [D] BG12-147, [E] BG15-40, [F] BG15-87, [G] BG16-787, [H] *E.coli* S-17 (negative control).

observation was noted in case of mushroom mycelia (Figure 2.12), indicating that the antifungal compound produced by BG164R is possibly distinct, and not involved in the expression of Cavity disease in mushrooms.

To further test this hypothesis, a genetically undefined mutant of BG164R with highly reduced capacity to inhibit fungal mycelia, BG110Af-, was tested for its ability to induce disease symptoms on mushroom slices. The mutant demonstrated the ability to cause cavity disease by this assay (Figure 2.13.A and B).

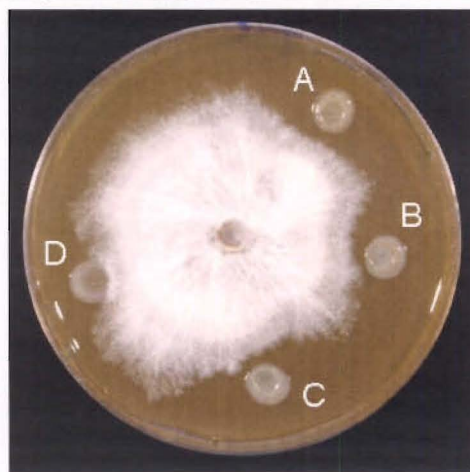


Figure.2.12: Inhibition of *Agaricus bisporus* mycelia by wild type BG164R, and representative 'no-cavity' causing mutants. [A] BG164R, [B] BG4-12, [C] BG12-147, [D] PA147-2 (-ve control)

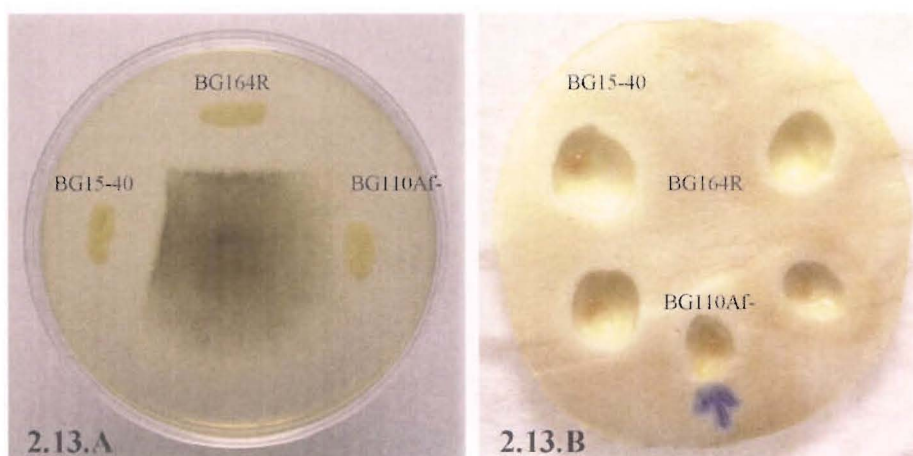


Figure 2.13: Antifungal [A] and mushroom bioassays [B] with antifungal reduced mutant (BG110Af-) and a representative no-cavity forming (BG15-40) mutant, in comparison to the wild type strain BG164R

2.4. Discussion.

In the experiments described above, the Cavity disease pathogen, BG164, was prepared for gene manipulation experiments, randomly mutated, and 'no-cavity' mutants were isolated using a mushroom bioassay system. In the process of preparing BG164 for gene manipulation experiments, initially the genetic markers present in the strain were defined by identifying its innate antibiotic resistance and susceptibility profiles. Thereafter, the spontaneous rifampicin-resistant mutant BG164R was further

mutagenised. The 6 prototrophic mutants derived from BG164R by mini-Tn5 insertion mutagenesis were found to differ from BG164R in regard to the flagella numbers, had a reduced capacity to degrade colloidal chitin, and lost the capacity to degrade protein on milk agar plates.

Growth curves were developed to compare the rate of growth of mutants to that of the wild type. All the prototrophic mutants isolated had similar rates of growth in the LB broth. The main objective of the growth experiments was to test whether capacity of growth of the mutants was linked to the inability of bacteria to cause cavity on mushroom slices, in the next set-up, the growth rates of a representative prototrophic and a representative auxotrophic mutant was monitored on mushroom slices over time. This clearly indicated that the population of the auxotrophic mutants gradually decreased on the slice of the mushroom tissues, while the population of the prototrophic mutants steadily increases over time. The 'no cavity' phenotype observed in case of the auxotrophic mutants could thus be due to the mutation itself, or due to the inability of the strain to grow on mushrooms. To identify the genes that had a role in the development of Cavity disease, it was decided to analyse the prototrophic mutants first.

Surface components in a bacterium often contribute to the virulence properties, besides serving as the main connection with the external environment (Stathopoulos *et al.*, 2000). Surface appendages like flagella and pili are essential for the colonisation of host surfaces, which serves as the initial stage in disease formation, both in the case of plants and animal pathogens (Lillehoj *et al.*, 2002; Tomich *et al.*, 2002; Chua *et al.*, 2003; Tans Kersten *et al.*, 2001; Ichinose *et al.*, 2003; Mulholland *et al.*, 1993). In *Vibrio cholerae*, flagella enable the bacterial cells to swim to intestinal mucosa in order to escape the peristaltic action of the intestine. Chua *et al.* (2003) in their study on the aflagellate, non-motile mutants of *B. pseudomallei* in the BALB/c mice model have demonstrated that flagella are virulence determinants. They facilitate the spread of *B. pseudomallei* throughout the respiratory tract and mediate adhesion to the host cells, but they do not have any role in the invasion of host cells. In contrast, flagella-mediated motility of *B. cepacia* has been proven to be the major contributory factor in the invasion of human alveolar epithelial carcinoma cell line A549 (Tomich *et al.*, 2002). In *P. aeruginosa*, flagella facilitates adhesion to the respiratory epithelial cells by binding

to a receptor of mucin, Muc1, secreted by the respiratory cells (Lillehoj *et al.*, 2002). In the case of plant pathogens like *Erwinia*, *Xanthomonas* and *P. syringae*, any mutation leading to aflagellate, non-motile phenotype has been demonstrated to be accompanied by the loss of pathogenicity (Ichinose *et al.*, 2003; Mulholland *et al.*, 1993; Tans Kersten *et al.*, 2001). Ichinose *et al.*, 2003 have reported that mutations in genes coding for flagellin protein, FliC, and the flagella cap protein, FliD, leads to reduced pathogenicity of *P. syringae* pv. *tabaci* on tobacco leaves and the mutants also had a reduction in the invasion capacity of the host tissue. In contrast, the pathogen *Ralstonia solanacearum*, requires flagella in the initial colonisation of the host by swimming towards to the host surface. Once internalised into the host tissue, flagella does not play any role in the production of disease symptoms (Tans Kersten *et al.*, 2001). Thus, flagella have been associated with pathogenicity of bacteria in different ways. All studies stated above have been analysed in light of the mutations that led to the loss of flagella formation in bacteria. The no-cavity forming mutants isolated in the course of this study were associated with the reduction in the flagella number and retained the capacity of migration very similar to that of the wild type. In *P. aeruginosa*, the *fleN* gene product regulates flagella number by acting as a negative regulator of *fleQ*, the transcriptional activator for the flagellar synthesising genes (Dasgupta *et al.*, 2000). Dasgupta *et al.* (2000) have demonstrated that in the *fleN* mutants of *P. aeruginosa* strains PAK and PAO1, motility was impaired although there was an increase in flagella number. Alternatively, there are reports of multiflagellate mutants of *P. aeruginosa* that swarm better. The mutations mapped to the *fla* locus of the chromosome which carries genes for basal body formation of the flagella (Suzuki, 1980). Dasgupta *et al.* (2000) proposed that phenotypes of motility and flagella number are not linked. Motility assay results presented in this chapter support the proposition of Dasgupta *et al.* (2000).

Flagella-mediated motility and chemotaxis forms a complex subsystem consisting of flagella and the sensory apparatus that controls its operation. The process of flagella biosynthesis and the controlled movement of the bacterial cells in response to environmental cues are controlled by a set of 40 genes in *E. coli* and *Salmonella typhimurium* spread over in 3 contigs within the bacterial chromosome (Macnab, 1992). Of these 40 gene products, the general secretory pathway secretes only two genes necessary for the formation of the flagella basal body while the flagella apparatus

secretes the rest. The two proteins secreted by the general secretory pathway of protein secretion in bacteria form the P and L rings in the basal body of flagella. The P and the L rings of flagella basal body have been associated with the maintenance of integrity of the rod in basal body structure and in no way linked to the possible control of the flagella number. Dasgupta *et al.* (2000) published the first report on the gene, *fleN*, controlling flagella number. Although majority of the genes in the complex flagella operon have been identified and the complex process of regulation of the different operons deciphered, the role of some genes still remain unknown. It has been presented in this chapter that the no-cavity mutants have a reduction in the number of flagella, while still retaining the capacity of motility. It is thus tempting to speculate at this stage that the mutants by some unknown mechanism have a role in the regulation of flagella number.

Interestingly, Cavity disease mutants in general do not show any difference in the capacity of biofilm formation under the defined assay system. One of the main features governing the formation of biofilms is availability of nutrients (O'Toole *et al.*, 2000). In the assay described, the media used was mushroom extract media, formulated specifically to best represent the nutritional conditions in the mushroom tissues. Apart from environmental cues regarding availability of nutrients, other factors have been associated with biofilm formation, such as possessing flagella, formation of type I and type IV pili, production of exopolysaccharides (O'Toole & Kolter, 1998b; Pratt & Kolter, 1998; Monds *et al.*, 2001). Common factors such as the ability of bacteria to swim and adhere to the target substratum and thereby multiply on them to form micro-colonies, have been found to be necessary in the formation of bacterial biofilms and in the virulence of pathogenic bacteria; however, the exact molecular mechanism involved in biofilm formation still is unknown. O'Toole and Kolter in their work on initiation of biofilm formation in *P. fluorescens* WCS365 (O'Toole & Kolter, 1998a) have demonstrated that extra cytoplasmic proteins take part in biofilm formation. In their rapid attachment assay they have demonstrated that in the presence of pronase E, the cells lose the capacity of adhering to abiotic surfaces. They have suggested that surface-attached proteins play a role in the biofilm formation, and hence in the presence of proteases, the initiation of biofilm formation is hampered. One of the phenotypic characters that distinguish the Cavity disease mutants from the wild type is the inability

to secrete proteases. However, no differences in the capacity of biofilm formation were observed in case of the mutants. Perhaps, inability to form biofilm in the presence of protease is a feature observed in case of only some specific proteases. Although there is a growing list of genes now known to be involved in biofilm formation, no study described so far has addressed the role of secretory proteases in the prevention of biofilm formation. From the results presented, it can be concluded that in the case of cavity disease, biofilm formation possibly does not play any significant role. Although, in order to confirm this hypothesis, biofilm formation by BG164R and the mutants on mushroom tissues need to be tested *in vivo*.

BG164R has a capacity to degrade colloidal chitin. Gill and Cole (1992) have reported the ability of BG164R to metabolise N-acetyl glucosamine, the monomer of chitin. The results presented in this chapter are consistent with Gill and Cole's observation. The mutants have a highly reduced ability to degrade colloidal chitin, in spite of not being able to form cavity on mushroom slices. Recently, a strain of *Burkholderia gladioli* (CHB101) that does not show any chitinase activity against colloidal chitin has been reported to produce chitosanase A. The enzyme hydrolyses glucosamine oligomers larger than pentamers (Shimosaka *et al.*, 2000), thus cannot degrade colloidal chitin on plate assays, which involves liberation of the monomers from oligomers and making them available to the bacteria to metabolise. Chemically chitin, is a β -1-4 linked polymer of N-acetyl glucosamine and for the bacteria to metabolise N-acetyl glucosamine, it has to make the monomer available in its immediate environment. Perhaps such sequential degradation of chitin takes place in case of BG164R, firstly hydrolysis of glucosamine oligomers into smaller units take place which is followed by the cleavage of the glycosidic bonds to liberate N-acetyl glucosamine, which is then metabolised by BG164R. Mutations interfere with one of the products involved in the sequential degradation of mushroom chitinase, as a result of which the mutants do not completely lose the capacity of degrading colloidal chitin Alternately, the mutations interfere with the secretion of chitin degrading enzymes of BG164R.

The bioassay system proved to be efficient for the purpose of screening mutant bacteria and testing an 'all or none' effect of cavity formation. However, slices obtained from different sporocarps varied in the intensity of disease manifestation under the

standardised assay conditions. Since colonies of the strain tested in the bioassay system were transferred onto mushroom slices with toothpicks, size of the initial inoculum used could possibly have been the explanation for such variation in the expression of disease symptoms. Alternatively, there could have been involvement of other intrinsic factor(s) in the expression of Cavity disease by BG164R on mushroom tissues, as will be discussed in chapter 5.

2.5. Concluding Remarks.

In summary, the mutants, in comparison to the wild type, have reduced number of flagella and have highly reduced capacity to degrade colloidal chitin, are unable to degrade milk proteins and associated with the shrinkage of mushroom mycelia. These could possibly imply that the same gene or different genes of a specific operon has been mutated in case of all the mutants. Identification of the gene(s) disrupted by transposon insertion in case of the mutants is most likely to provide possible explanations of the phenotypes noticed in the mutants. Thus, identification of the mutated genes was essential. The next chapter describes the cloning of mutants and identification of the genes that have rendered the pathogen avirulent.

Chapter III

Identifying the gene(s) of BG164R involved in the manifestation of Cavity disease symptoms.

3.1. Introduction.

Cavity disease is a 'soft rot' disease of the mushroom sporocarp. As such, BG164R is expected to produce and secrete mushroom cell wall depolymerisation enzymes. The characteristic difference between the cell walls of plants and mushrooms is the presence of specific components like chitin and glucan, which make up the skeletal structure of the mushroom mycelia (Alexopoulos, 1996). The observed phenotypic differences (presented in section 2.3.7, 2.3.11 and 2.3.12) between the wild type and mutants of BG164R isolated in the mutagenesis screens were a reduction in the flagella number, inability to degrade protein on milk agar plate and highly reduced capacity to degrade colloidal chitin. Identification of the genes mutated in the avirulent derivatives of the pathogen appeared to be the best approach to relate the genetic mechanism(s) of BG164R involved in the expression of Cavity disease.

This chapter describes the cloning of chromosomal regions to which these mutations map. Cloned genes adjacent to the insertion points of the transposon were sequenced. A genomic library of BG164R was constructed; putative cosmids capable of complementing the null mutants were isolated and used to complement the mutants. Attempts made to identify the active products involved in Cavity disease are also described. In the last section, the role of BG164R protease(s) in Cavity disease manifestation is discussed.

3.2. Material and Methods.

3.2.1. Bacterial strains, plasmids and cosmids either created or used in the course of the study.

Strains and plasmids	Genotype or description	References
<i>Burkholderia gladioli</i> pv. <i>agaricola</i> .		
BG164R	wildtype, cav+, Af+, Rif ^R , Prot+	This study
BG4-12	BG164R <i>gspF</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG12-88	BG164R <i>gspK</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG15-40	BG164R <i>gspE</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BGProt-123	BG164R <i>gspL</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BGProt-125	BG164R <i>gspF</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG4-12Cos	BG4-12 complemented with cosmid pCosGSP, Rif ^R Kan ^R , Tet ^R ,cav ⁺	This study
BG12-88Cos	BG12-88 complemented with cosmid pCosGSP, Rif ^R Kan ^R , Tet ^R ,cav ⁺	This study
BG15-40Cos	BG15-40 complemented with cosmid pCosGSP, Rif ^R Kan ^R , Tet ^R ,cav ⁺	This study
BG-LAF ₃	BG164R with pLAFR ₃ , Rif ^R ,Tet ^R , cav ⁺ , Prot+	This study

BG4-12LAF ₃	BG4-12 with pLAFR ₃ Rif ^R , Kan ^R , Tet ^R , cav ⁻ , Prot-	This study
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Eschericia coli

DH5α	<i>supE44 ΔlacU169 (ϕ80lacZΔM15) hsdR17 thi-1 relA1 recA1</i>	Hanahan 1983
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HB101	<i>supE44 hsdS20 (r_B-m_B-) recA13 ara-14 rspL20 proA2 lacY1 galK2 xyl-5 myl-1</i>	Boyer 1969
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Plasmids and constructs

pSPRC12	8.5kb <i>Sall</i> fragment containing <i>gspF</i> ::mini-Tn5 KmlacZ2 from BG4-12 in pBluescript KS+, Ap ^R , Kan ^R .	This study
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pSPRC40	5.4kb <i>Sall</i> fragment containing <i>gspE</i> ::mini-Tn5 KmlacZ2 from BG15-40 in pBluescript KS+, Ap ^R , Kan ^R .	This study
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pSPRC87	6.0kb <i>Sall</i> fragment containing <i>gspD</i> ::mini-Tn5 KmlacZ2 from BG15-87 in pBluescript KS+, Ap ^R , Kan ^R .	This study
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pSPRC88	5.2kb <i>Sall</i> fragment containing <i>gspK</i> ::mini-Tn5 KmlacZ2 from BG15-88 in pBluescript KS+, Ap ^R , Kan ^R .	This study
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pSPRC147	5.3kb <i>Sall</i> fragment containing <i>gspK</i> ::mini-Tn5 KmlacZ2 from BG12-147 in pBluescript KS+, Ap ^R , Kan ^R .	This study
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pSPRC787	5.4kb <i>Sall</i> fragment containing <i>gspE</i> ::mini-Tn5 KmlacZ2 from BG12-787 in pBluescript KS+, Ap ^R , Kan ^R .	This study
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pBGProt-123	6.6kb <i>Sall</i> fragment containing <i>gspL</i> ::mini-Tn5 KmlacZ2 from BGProt-123 in pBluescript KS+, Ap ^R , Kan ^R .	This study
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pBGProt-125	8.3kb <i>Sall</i> fragment containing <i>gspF</i> ::mini-Tn5 KmlacZ2 from BGProt-125 in pBluescript KS+, Ap ^R , Kan ^R .	This study
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pROBE	1.5kb <i>NotI</i> fragment containing the kanamycin gene from mini-Tn5Km/ <i>lacZ</i> 2 blunt end cloned into the <i>EcoRV</i> site of pBluescriptKS-;Ap ^R	S.R.Giddens
pCosGSP	23.4kb <i>Sau3AI</i> partial fragment containing GSP gene cluster from BG164R into pLAFR ₃ .	This study
Vectors.		
pBluescript (KS,M13+)	ColE1 <i>ori lacZα</i> /KS polylinker, T3/T7.Ap ^R	Stratagene
pBR322	ColE1 <i>ori</i> Ap ^R Tc ^R .	Bolivar <i>et al.</i> 1977
pLAFR3	pRK290 derivative;RP4(IncP-1) <i>ori</i> , λ <i>cos</i> , pUC9 multicloning site and <i>lacZα</i> ,Tc ^R	Staskawicz <i>et al.</i> 1987

Table 3.1. List of Strains used in the experiments described in Chapter 3.

3.2.2. Media, reagents and antibiotics.

All media and reagents used in this study were prepared as described in Appendices I and II. Concentrations of the antibiotics used to maintain selective pressure on either strains or plasmids/cosmids within a strain are as described in Appendix I, unless otherwise stated.

3.2.3. Bacteriological methods and mushroom bioassay system.

The bacteriological methods and the culture conditions used are stated in section 2.2.3. All mushroom bioassays were carried out according to the method stated in section 2.2.5.

3.2.4. DNA manipulation and cloning techniques.

3.2.4a. Preparation of Genomic DNA (Pitcher *et al.* 1989)

The method used was adopted from Pitcher *et al.* (1989), whereby 1.5 ml liquid cultures of 16hr old cells were harvested by centrifugation at 7,000rpm for 2min and the pellet was resuspended in 1/15th vol (100µL) of Tris-EDTA buffer. Bacterial lysis was mediated by the addition of 5 volumes (500µL) GES solution (Appendix II), followed by thorough mixing and incubation at 65° C for 15min. Cold 7.5M ammonium acetate (Appendix II) was added, at half the volume of the GES added (250µL), and the solution mixed thoroughly before being incubated on ice for 10 min. Proteins were extracted by addition of double the volume of ammonium acetate (500µL) of chloroform: isoamyl alcohol (24:1). Aqueous and organic phases were mixed and separated by centrifugation at 12,000rpm for 3min. DNA was precipitated from the aqueous phase by addition of 0.54 volumes of isopropanol, mixing with care by inversion and incubation at room temperature for 15min. The dehydrated filaments of DNA were recovered by centrifugation, rehydrated by washing thrice with 70% ethanol, air dried and resuspended in 50µL of T₁₀E₁ Buffer (Appendix II).

3.2.4b. Plasmid DNA preparation (Sambrook *et al.*, 1989)

Plasmid DNA was prepared by the standard alkaline lysis method derived from Brinboim and Dolly (1979), as stated by Sambrook *et al.* (1989). For routine analysis of plasmid DNA, cells from 1.5mL of a 16-18hr old culture were harvested by centrifugation at 7,000rpm for 2min at room temperature and the supernatant was removed by aspiration. The cells were resuspended in 1/15th volume (100µL) of solution I (Appendix II) and incubated for 10min on ice. Double the existing volume of freshly prepared solution II (Appendix II) (200µL) was added and mixed gently until the solution became viscous and clear, indicating complete lysis of cells had occurred. Following this, half the total existing volume of ice-cold solution III (Appendix II) (150µL) was added and mixed vigorously until a white precipitate appeared. The solution was incubated on ice for 10min, followed by centrifugation for 10min at 13,000rpm at 4°C. The supernatant was decanted into a fresh Eppendorf tube, and half

the existing volume of ice-cold isopropanol (250 μ L) was added in order to dehydrate the DNA molecules. After incubation on ice for further 10min the DNA was precipitated and collected at 13,000rpm for 10min at 4°C. The DNA pellet was washed once with 70% ethanol at RT, which partially re-hydrates DNA. The DNA was then air-dried and resuspended in 200 μ L of T₁₀E₁ buffer. Double the volume (400 μ L) of 100% ice-cold ethanol was added, to re-precipitate the DNA, and held on ice for 10 min before centrifugation at 13,000 rpm at 4°C for 10min. The DNA collected was washed thrice in 70% ethanol, air-dried and resuspended in sterile distilled water. The volume of the sterile distilled water used to resuspend the DNA extracted, depended on the copy number of the plasmid DNA being extracted. For high copy plasmid DNA like pBluescript and its derivatives, the DNA was generally resuspended in 50 μ L of T₁₀E₁, for medium copy number plasmid like pBR322 or low copy number pLAFR₃ based cosmid constructs, 20 μ L T₁₀E₁ was used. DNA concentration was estimated (3.2.4c) and the working DNA solutions were normally diluted to a concentration of approximately 500ng/ μ L before being stored at -20°C.

3.2.4c. Estimation of DNA concentration (Sambrook *et al.*, 1989).

The concentration of various DNA samples was determined by measuring the ratio of absorbance at 260nm relative to the absorbance at 280nm using a deuterium lamp LKB Ultraspec[®] Plus spectrophotometer. DNA was generally diluted to 1:100 and the relative absorbance measured. The concentration of the DNA sample was calculated from the standard $1\text{OD}_{260} = 50\text{ng}$ of nucleic acid.

3.2.4d. Preparation of sequencing/cloning quality DNA (Sambrook *et al.*, 1989).

An essential criterion in sequencing or cloning reactions is the quality of the DNA template. Thus, plasmid DNA prepared by the alkaline lysis method (stated in section 3.2.4b) and required for further manipulation was re-suspended in 200 μ L of T₁₀E₁ and subjected to further purification. This included a lithium chloride treatment for the precipitation of high molecular weight RNA and an RNase treatment for the elimination of low molecular weight RNA, followed by a phenol chloroform extraction to remove all protein contaminants.

Briefly, to 200 μ L of DNA (extracted by the alkaline lysis method) an equal volume of ice-cold 5M LiCl₂ solution was added. The solution was mixed and incubated on ice for 10min followed by centrifugation at 13,000rpm, 10min at 4°C. The supernatant, containing the DNA and small molecular weight RNA molecules, was transferred to a fresh tube. An equal volume of ice-cold isopropanol was added, followed by incubation for 10min on ice. The nucleic acid was collected by centrifugation (13,000rpm, incubated on ice for 10min at 4°C), rinsed in 70% ethanol at RT, air dried and re-suspended in 200 μ L of T₁₀E₁. RNA was removed by the addition of 2 μ L of 10mg/mL RNase solution and incubation at 37°C for 25min. The DNA was subsequently purified by adding equal volumes of phenol and chloroform:isoamyl alcohol. The mixture was centrifuged to separate the aqueous and the organic phases at 12,000rpm for 3min at RT. The aqueous phase, containing the DNA, was collected and the DNA was precipitated by adding 1/10th volume of 3M sodium acetate and 2 volumes of 100% ice-cold ethanol. The solution was mixed well and incubated on ice for 10min. The DNA was recovered by centrifugation at 13,000rpm for 10min, washed thrice in 70% ethanol at RT and re-dissolved in sterile distilled water to give a final concentration of 100-500ng/ μ L.

3.2.4e. Restriction digestion of DNA.

Restriction digests were set up according to the manufacturer's recommendations. Digestions were routinely carried out in a final volume of 10 μ L and incubated at 37°C for 1-3hr. For digestions that required more than one restriction enzyme, a buffer best suited for all the enzymes were used. Alternatively, the digestion was carried out in more than one step, separated by thermal denaturation of the enzyme at 80°C for 15min and precipitation of the DNA.

3.2.4f. Agarose gel electrophoresis of DNA(Sambrook *et al.*, 1989).

DNA fragments digested with restriction enzymes were analysed by agarose gel electrophoresis. The agarose gel was prepared with 1 x TAE buffer (Appendix II) at a concentration varying from 0.5% to 0.9% depending upon the size of the expected DNA fragments to be separated. The resolved DNA fragments were stained for 10min with 0.5ng/mL ethidium bromide solution prepared in 1 x TAE. Generally, an aliquot of λ DNA digested with *Hind*III was used as the standard against which the size of the DNA

fragments being electrophoresed were estimated. Following the staining of DNA, gels were destained in 1 x TAE for 5 mins before visualisation at a wavelength of 256nm on a Sigma T2210 UV trans-illuminator. The DNA gels were photographed using the Kodak Electrophoresis Documentation and Analysis System 120.

3.2.4g. Elution of DNA from agarose gels.

The desired DNA fragments were eluted from agarose gels with the “Prep-a-Gene” DNA purification kit from Bio-Rad® using the manufacturer’s protocol. The DNA was eluted from the matrix using sterile distilled water.

3.2.4h. Calf intestinal phosphatase (CIP) treatment of DNA (Sambrook *et al.* 1989).

To enhance the chances of ligation of insert DNA to the vector, unless otherwise stated, the vector DNA was treated with calf intestinal phosphatase (CIP) prior to ligation. The basic principle of the reaction is removal of the terminal 5’ phosphate from the digested DNA by treatment with phosphatase. This reduces the chances of self-ligation of the vector DNA.

The DNA to be CIP treated was precipitated and re-suspended in 49µL of 1 x dephosphorylation buffer (from the 10 x stock provided by Böehringer Mannheim) and 1 unit of CIP (Böehringer Mannheim). The reaction was incubated at 37°C for 30min before being terminated by addition of EDTA (Appendix II) pH 8 (to make final concentration of the solution 5mM) at 75°C for 10min. Following the required incubation period, the volume of the DNA was made up to 200µL, and extracted with phenol and chloroform using the method stated in section 2.3.4d. The DNA was re-dissolved in 10µL of sterile distilled water.

3.2.4i. DNA ligation (Sambrook *et al.*, 1989).

Estimated quantities of the vector and insert DNA, in the ratio of 1:3 respectively (unless otherwise stated), were transferred into an Eppendorf tube and precipitated by addition of 1/8th volume of 2M potassium acetate pH 8, and double the volume of 100% ice-cold ethanol. The mixture was incubated at –80°C for 20min and the DNA recovered by centrifugation at 13,000rpm for 10min at 4°C. The precipitated DNA was

re-suspended in 11 μ L of sterile distilled water, heated at 45°C for 5min and crash cooled to separate any annealed cohesive end ligation. To this 3 μ L of 5 x ligation buffer and 2 units of T4DNA ligase (GIBCO BRL) was added and incubated at RT for 16hr (unless otherwise stated). At the end of the required incubation period, an aliquot was run on an agarose to check the efficiency of ligation, re-precipitated with 2M potassium acetate as stated above and dissolved in sterile distilled water at a final concentration of 50ng/ μ L.

3.2.4j. Cloning of transposon tagged genes from the genomic DNA of BG164R (Sambrook *et al.*, 1989).

Generally, 8 μ g of genomic DNA and 2 μ g of vector DNA were digested to completion with 5 units and 1 unit of the appropriate enzyme respectively. Following digestion, an aliquot of both the insert and vector DNA was checked on an agarose gel, the enzymes were thermally denatured, and the final volume of DNA was made up to 200 μ L with sterile distilled water. DNA was precipitated by the addition of 1/10th volume of 3M sodium acetate, double the final volume of 100% ice-cold ethanol, and incubated at – 80°C for 20 min prior to centrifugation at 13 000rpm for 10 min at 4°C. This DNA served as the digested stock, and ligation was set up with 2 μ g of insert DNA and 500ng of vector DNA for 18 to 20hr at room temperature with 4 units of T₄ DNA ligase.

Following ligation for 20hr, aliquots of the ligated DNA were checked on a 0.9% agarose gel prior to re-precipitation as described above with sodium acetate. Approximately, 100ng of ligated DNA was used for electro-transformation of *E. coli* DH5 α electro-competent cells according the method stated in section 3.2.5.

3.2.5. Electro-transformation. (Dower *et al.* 1988; Zabarovsky and Winberg 1990).

3.2.5a. Preparation of Electro-competent *E.coli* cells.

The method used was as recommended by the Bio-Rad pulse controller instruction manual. One hundred mL of an exponentially growing culture of *E. coli*, starting from a 1:100 dilution of 18hr old stationary phase culture, was grown to an OD₆₀₀ of 0.5 to 0.7. The culture was chilled on ice for 30min and cells were harvested by centrifugation at

5,000rpm for 5min at 4°C. The cells were washed by resuspending in 100mL of ice-cold sterile distilled water and centrifugation at 5,000rpm for 5min at 4°C. This was followed by a second wash in half the volume of ice-cold sterile distilled water and a third wash in one fourth volume of ice-cold 10% sterile ice-cold glycerol. The cells were thereafter resuspended in 500µL of 10% sterile ice-cold glycerol, and 40µL aliquots were transferred to sterile and cold Eppendorf tubes. The cells were either freshly used or stored in –80°C for use up to 3months.

3.2.5b. Electroporation of ligated DNA into competent *E. coli* cells.

Electro-transformation was carried out by electroporating the competent cells with the recombinant DNA using a Gene Pulser™ (Bio-Rad) set with capacitance at 25µF, and a pulse controlled resistance of 200Ω. For cuvettes with 0.1cm gap the voltage were set at 1.8kV and for 0.2cm gap cuvettes, the voltage was set at 2.5kV. Generally, freshly prepared electro-competent cells were used. When frozen cells were used in the cloning experiments, the frozen cells were thawed on ice for 20min prior to transformation reaction. Sterile and cold cuvettes as well as the slide chamber were mandatory for electro-transformation reactions. Usually 50-100ng of DNA was mixed with the electro-competent cells, transferred in pre-cooled sterile cuvettes, incubated for 5min before exposure to the electric pulse set with conditions mentioned above. Immediately after the charge release, the cells were retrieved from the cuvette with 1mL of SOC media (Appendix I). The transformed cells were elaborated for 1hr at 37°C, centrifuged for 2min at 7,000rpm and harvested cells were spread on LB plates supplemented with appropriate antibiotics and incubated at 37°C for 24hr.

3.2.6. Southern Hybridisation (Sambrook *et al.*, 1989).

3.2.6a. Probe labelling.

A 1.5kb *NotI* fragment containing kanamycin gene from mini-Tn5KmlacZ2, was cloned in pBluescriptKS+ to construct pROBE (Table 3.1.). This was used to probe *SaI* digested genomic DNA of the mutants to verify single transposon insertions. Random Primed DNA labelling kit from Böehringer Mannheim was used to radioactively label

50ng of the probe DNA, following the manufacturer's protocol. (αP^{32}) dCTP was used as a labelled nucleotide, and the labelling reaction was incubated at 37°C for 30min.

3.2.6b. Transfer method.

The digested DNA was separated on a 0.9% agarose gel at 16 Volts for 12hr. The gel was stained briefly in freshly made TAE solution containing ethidium bromide (0.5 $\mu\text{g}/\text{mL}$), visualised and photographed prior to destaining for an hour. The DNA was transferred from the gel on to a HybondTM N+ membrane using a Pharmacia LKB VacuGene XL vacuum blotting apparatus and following the protocol supplied in the instruction manual. This included partial depurination of the DNA by covering the gel with 0.25M HCl for 15min under 50mbar pressure, followed by replacing the HCl with 4M NaOH for the denaturation and transfer of the DNA under similar pressure for one hour. Following transfer, the gel was restained to confirm complete transfer of DNA to the membrane and the membrane was washed briefly in 2 x SSC before probing.

3.2.6c. Hybridisation and signal detection.

The membrane was placed in a Hybaid glass hybridisation tube and pre-hybridised in the recommended pre-hybridisation buffer for 3hr at 65°C prior to addition of the labelled probe, in a rotary mini-hybridisation oven. Hybridisation was carried on for 18hr at 65°C. Following hybridisation, the buffer was replaced with 2 x SSC and washed for 15min to remove unbound probe. The stringency washes were thereafter done at 65°C to ensure removal of non-specifically bound DNA probes. Stringency washes consisted of washing the membrane thrice in 2 x SSC and 0.1% SDS for 5min each, followed by two washes for 10min each in 1 x SSC and 0.1% SDS, and four washes in 0.1% SSC and 0.1% SDS, 5min each. The washed membranes were taken out of the rotary tube, wrapped in a Gladwrap and exposed to Amersham Hyperfilm-MP in an autoradiography cassette for 2hr at -80°C. The film was immersed in Agfa G-150 developer for 5min, rinsed in flowing tap water and fixed in Agfa G-334 fixer for further 5min. The autoradiograph was then rinsed in water and air-dried.

3.2.7. DNA Sequencing.

The chain terminating dideoxynucleotide triphosphate method of Sanger (1977) was used to sequence the DNA using infrared-labelled T3 and T7 primers. Initially all the clones were sequenced using a LI-COR4000L automated DNA sequencer in the School of Biological Sciences. The clones were later re-sequenced at the DNA sequencing facility of the University of Auckland using the same set of primers and using a LI-COR 4000LD IR2 automated sequencer using two infrared dyes IRD700 and IRD800. The template DNAs were supplied to them at a concentration of 0.2pmol/ μ L in sterile distilled water. The sequencing reactions and the gel conditions used for sequencing the clones in the School were as follows:

3.2.7a. Sequencing reactions.

The SequiTherm™ Long-Read™ Sequencing Kit LC from Epicentre Technologies was used for sequencing. The primers used were T3 and T7 labelled IRD41 dye. The reaction mixtures were made up following the manufacturer's protocol provided with the kit. Briefly, 0.5pmol template DNA was added to 2.5 μ L sequencing buffer, 1 μ L of DNA polymerase, 2pmol of IR-labelled primer and the final volume of the mixture was made up to 17 μ L with sterile distilled water. 4 μ L aliquots from this bulk reaction mix was added to 4 separate 0.5mL PCR tubes containing 2 μ L of 4 different Long-Read™ termination mixes (of one ddNTP and the remaining 3 dNTPs per mixture). This reaction mixture was overlayed with a drop of PCR mineral oil to prevent evaporation during the cycling reactions. The contents of the tube were collected at the bottom of the tube by a quick spin in a bench top centrifuge. The PCR reactions were done in a Hybaid Omnigene thermocycler for 30 cycles of 30sec at 95°C, 15sec at 50°C and 1min at 70°C. At completion, the reactions were terminated by adding 3 μ L of stop solution containing formamide, EDTA and fuschin loading dye. The reaction mixes were immediately loaded on the sequencing gel and run overnight for 16hr, unless otherwise stated. Excess reaction mixes were stored at -20°C. Immediately before loading of the samples, the samples were denatured by heating for 5min at 95°C.

3.2.7b. Sequencing gels.

Sequencing of double stranded DNA was done in a Li-COR automated sequencer available in the department, exploiting Sangers' dideoxy chain termination method (Sanger *et al.*, 1977). A 4% Long Ranger Gel solution (Bio Whittaker Molecular Applications) was used to cast the gel using the casting mixture mentioned in Appendix II. Sixty six cm long gel casting plates were used with a 32 well comb. Polymerisation was carried out by incubation at RT for 2-3hr. Prior to loading the samples, the gels were pre run in 1 x TBE (Appendix II). Data obtained from the electrophoresis of the samples were collected by using 2.31 Data Collection DEV7 software and the data were recorded using Version 2.30 Image Analysis Program of the LI-COR4000L automated DNA sequencer.

The sequences obtained were confirmed by re-sequencing at the University of Auckland DNA sequencing facility. Sequences collected from both the sources were aligned using the software, DNA MAN Version 4.02 and is presented in Appendix III.

3.2.7c. Analysis of sequence data.

Sequence data obtained were first analysed to find overlapping sequences of the vector and the transposon ends using DNAMAN Version 4.02 program from Lyonnnon BioSoft. The sequences were processed to separate the overlapping sequences from the genomic DNA sequences. The processed sequences were thereafter submitted to BlastN and BlastX servers (www.ncbi.nlm.nih.gov/blast) and compared to the other referenced sequences in the database following methods stated by Altschul *et al.*, (1997).

3.2.8. Construction of genomic library of BG164R (Fleischman *et al.* 1987).

The genomic library was constructed according to the method outlined by Fleischman *et al.* 1987, except for the extraction of genomic DNA, which was done according to method stated by Pitcher *et al.* 1989 and described in section 3.2.4a.

3.2.8a. Preparation of BG164R insert DNA.

(i) Digestion of genomic DNA to obtain partially digested 20 kb fragments.

Twenty sets of restriction digestions, each containing 25µg of genomic DNA of BG164R were digested with 0.005U enzyme/µg of DNA, for 7.5min to obtain 20-25kb partially digested DNA fragments. Aliquots from each digest were analysed on a 0.4% agarose gel, run for 14hr at 12 Volts with *Hind*III digested λ-DNA as a molecular weight marker. Digested DNA samples were pooled and precipitated with 1/10th volume of 3M Na-Acetate and twice the volume of cold 100% ethanol, washed thrice with 70% ethanol, dried and re-dissolved in 20µL of T₁₀ E₁.

(ii) Sucrose density gradient centrifugation for size fractionation of the genomic DNA.

Partially digested genomic DNA fragments (20-25kb) were purified by sedimentation through a 10-40% (w/v) continuous sucrose density gradient, following the method stated by Sambrook *et al.* (1989) with a few modifications.

- (a) DNA was centrifuged in a SW27 rotor for 24hr at 26,000 rpm at 15°C.
 - (b) Two hundred and fifty µg of DNA was loaded per 38ml gradient made in 40ml Beckman ultracentrifuge tubes.
 - (c) One ml fractions were removed from the top of the gradient and 10µL aliquots of every second fraction was separately analysed on a 0.4% agarose gel run at 12Volts for 14hr with *Hind*III digested λDNA as the molecular weight marker.
 - (d) Following isolation of the 1mL fractions, which could be used in the library construction, each of them was separately diluted thrice with T₁₀E₁. The pH was adjusted with 1/10 volume of 3M Na-acetate pH-4.8, precipitated with twice the volume cold 100% ethanol, and resuspended in 20µL of T₁₀E₁.
 - (e) One µL from each fraction was loaded and finally analysed on a 0.4% agarose gel run at 12Volts for 14hr. Desired fractions were there after pooled into one
-

and the concentration of the insert DNA was estimated according to the method stated in section 3.2.4c.

3.2.8b. Preparation of pLAFR₃ vector DNA.

(i) Extraction and purification of vector DNA.

pLAFR₃ DNA was prepared by the standard plasmid DNA preparation method by alkaline lysis, as described in section 3.2.4b. from 2L culture of *E. coli* cells harbouring the cosmid. Extracted cosmid was purified by CsCl₂-ethidium bromide equilibrium centrifugation method described in Sambrook *et al.* (1998), with a few modifications in the methods of removal of ethidium bromide from the DNA isolated.

- (a) An equal volume of isopropanol, saturated with CsCl₂, was added to the band of DNA ethidium bromide CsCl₂ solution taken out from the Beckman ultracentrifuge tube with a 16-gauge hypodermic needle and mixed by inversion.
- (b) The red, ethidium bromide containing, upper layer was discarded and the DNA solution was washed twice with isopropanol saturated CsCl₂.
- (c) After the second wash, the CsCl₂ DNA solution was put in a micro-concentrator tube and spun at 5,000 rpm for 20min at 4°C. This was repeated 3 times to remove the CsCl₂.
- (f) The concentration of the DNA solution thus obtained was estimated according to the method stated in section 3.2.4c.

(ii) Digestion and CIP treatment of the Vector DNA.

Purified pLAFR₃ DNA was digested with *Bam*HI according to the conditions stated in section 3.2.4e., in this case to ensure the quality of DNA prepared and to ensure the complete linearisation of vector DNA. Linearised DNA was ethanol precipitated and resuspended in T₁₀E₁. The vector was dephosphorylated by CIP treatment following the method stated in section 3.2.4h, and the concentration of DNA estimated according to the method stated in section 3.2.4c.

3.2.8c. Library construction.

(i) Optimisation of the conditions for the digestion of genomic DNA.

In a series of preliminary reactions, ideal conditions best suited to obtain 20-25kb partially digested fragments of 1µg genomic DNA were established by varying the time of digestion and enzyme (*Sau3AI*) concentrations in the reaction. Aliquots from each digest set up with varying parameters were analysed on a 0.4% agarose gel run for 14 hr at 12 Volts with *HindIII* digested λ-DNA as the standard molecular weight marker. From these reactions, optimum conditions of 7.5min digestion with 0.005U enzyme /µg of DNA was chosen. To set up digests using 25µg of genomic DNA per reaction, all reagents were scaled up and reaction conditions duplicated. Prior to size fractionation of DNA by sucrose density gradient centrifugation, 20 sets of scaled up digestions were set up and each digest was analysed separately on 0.4% agarose gel to ensure presence of the desired fragment sizes of partial digests in the reaction mix. The ideally digested DNA samples were pooled together, precipitated, and purified by sucrose density gradient centrifugation.

(ii) Preparation of Vector DNA.

Initially, 1µg of purified pLAFR₃ DNA was digested with *BamHI*, to ensure the quality of the DNA prepared and to establish the digestion conditions. This was followed by 5 sets of bulk digestion each containing 6µg of DNA and replicating the conditions established. Complete linearisation of vector DNA was ensured by running an aliquot from each digest on a 0.9% agarose gel at 80 Volts for 2hr. Linearised DNA was precipitated with ethanol and resuspended in T₁₀E₁ before being dephosphorylated by CIP treatment, following the method described in section 3.2.4h. The dephosphorylated vector DNA was extracted with phenol: chloroform to terminate the enzyme activity and remove contaminants of the reaction mixture, precipitated with ethanol and stored as the stock.

Dephosphorylation of the 5' ends in the vector molecule was of paramount importance in the construction of a genomic library to avoid ligation of vector's arms leading to wrongly packed phage heads. Thus, before ligation of the vector with the insert DNA,

the efficiency of dephosphorylation was tested by comparing ligation reactions set up with 100ng of dephosphorylated vector and control non-dephosphorylated vector molecules independently. This was followed by transformation of electro-competent *E. coli* DH5 α cells with 100ng DNA from the two above mentioned ligation mixtures. Results indicated a 98% efficiency of CIP treatment of the vector DNA. The final concentration of dephosphorylated linearised vector DNA was estimated on a LKB Ultraspec Plus spectrophotometer and found to be 100ng/ μ L of the DNA solution. The insert and the vector DNA were ligated and packaged into phage heads using the Promega packaging kit and following the manufacturers protocol as described in section 3.2.8.

(iii) Ligation of vector to the insert DNA.

Ligations were set up using a 3:1 vector to insert DNA ratio (900ng of vector and 300ng of insert DNA) to ensure head on ligations of the vector and insert DNA instead of forming ccc-recombinant DNA molecules. The head on ligation would facilitate packaging of DNA into the phage heads. The ligations were set up for 4 hrs at RT with 3 units of T₄DNA ligase, run and analyzed on a 0.4% agarose gel.

(iv) *In vitro* packaging of the Library into Phage heads.

Ligated DNA was packaged *in vitro* into the phage heads by using Packagene[®] 'Lambda DNA Packaging System' packaging extract bought from Promega, and using the manufacturer's protocol (Promega Technical Bulletin 005). Briefly, the 1.2 μ g ligated DNA was added to 50 μ L of the packaging extract and incubated at room temperature for 3hr. A negative control for the packaging reaction was carried out by packaging of 900 μ g of non-ligated vector DNA in 25 μ L of Packaging extract. A positive control to test the efficiency of packaging was carried out by packaging 488 μ g of Packagene[®] control DNA (c1857 *Sam7*), provided in the kit.

To determine the efficiency of packaging, a permissive host LE392 was made susceptible to phage infection. A serial dilution of the packaged positive control DNA (provided in the kit) was made. These were used to infect LE392 made susceptible to phage infection, in order to estimate the phage titre.

(v) Optimisation of conditions for transduction of DH5 α recA- strain to construct the cosmid library.

Dilutions of the packaging mix were used to transduce DH5 α recA- cells. In a series of preliminary reactions 1:10 and 1:100 dilutions of packaging extract were used to transduce 100 μ L and 200 μ L of susceptible DH5 α cells. These reactions showed that the most efficient rate of transduction was obtained by transducing 100 μ L of susceptible DH5 α cells with 50 μ L of the 1:10 dilution of packaging extract. Cosmid DNA was prepared from 10 transductants, digested with *Eco*RI and *Bam*HI and run on a 0.9% agarose gel to check the nature and sizes of the insert. All 10 cosmids had different restriction pattern, and for each digest the total size of the fragments of insert DNA was approximately 20Kb.

(vi) Transduction of DH5 α recA- strain to obtain the cosmid library.

DH5 α recA- cells were made susceptible to phage infection by growing the cells in maltose supplemented LB broth for 20hr, followed by harvesting of the cells by centrifugation at 7,000rpm for 3min at RT and resuspending the pellet in half the volume of 10mM MgSO₄.

To construct the genomic library, 100 μ L of susceptible DH5 α cells were mixed with 50 μ L of the 1:10 dilution of packaging extract and incubated at RT for 30min. The mixtures were thereafter diluted and spread on LB plates supplemented with tetracycline to select for DH5 α clones containing pLAFR₃ derived cosmids.

3.2.8d. Amplification and storage of the library.

Six thousand colonies arising from 5 independent transduction experiments carried out with the standardized ratios and stored. The transduced cells were collected from the plates with 3mL of terrific broth (TB) (Appendix I), washed with an additional 2mL of terrific broth, pooled together, volume of the final terrific broth was made up to 50mL and incubated for 3hr at 37°C. After the incubation, the cells were spun down, resuspended in 40ml of TB supplemented with 15% glycerol. 500 μ L aliquots were stored in the -80°C freezer.

3.2.9. Colony Hybridisation.

3.2.9a. Colony transfer.

Three thousand and two hundred individual library clones were replica toothpicked on to 90mm LB plates supplemented with tetracycline. One of the plates from each replica was overlayed with a Hybond™ N+ membrane, so as to cover all the colonies toothpicked onto the plate and incubated at 37°C for 18hr, while the master plate was stored at 4°C. Following adequate incubation period of the replica plate lined with the membrane. The membrane was marked by making a hole through the membrane into the agar surface. This facilitated aligning of the autoradiogram with the plates later on. A pair of sterile forceps was used to remove the membrane, which was processed for the binding of DNA liberated from each lysed colony on the nitrocellulose membrane, as described in the following section.

3.2.9b. Binding of DNA to the membrane.

The membrane containing the colonies were placed in a tray containing 3mm filter paper flooded with 10% SDS (Appendix II), with the side of the membrane containing the colonies facing up, and incubated for 3min to facilitate colony lysis. The membrane was removed from the solution and placed on paper towels to drain the excess SDS solution. Thereafter, the membrane was transferred to a filter paper flooded with denaturing solution (Appendix II) and incubated for 5min. The membrane was again air dried by placing on a paper towel. This was followed by treatment of the filter paper with a neutralising solution (Appendix II) for 5min. The neutralising reaction was repeated, after which the membranes were allowed to dry in air for 10min. After complete drying, the membranes were pre-washed in 3 x SSC, 0.1% SDS at 65°C for 2-3hr, followed by rinsing in 2 x SSC, to remove excess debris.

3.2.9c. Probe labelling.

The probes were labelled according to the protocol described in section 3.2.6a. To retrieve the cosmids containing intact copies of *gsp* genes of BG164R, a probe was constructed by eluting the 1.5 kb *EcoRI/SaII* fragment of genomic DNA from T3 end of the mutant clone, pSPRC12 (Fig.3.2).

3.2.9d. Hybridisation and Stringency washes.

The membranes were placed in Hybaid hybridisation tubes and prehybridised in prehybridisation buffer (Appendix II), for 3hr at 65°C prior to addition of labelled probe (approx. 200K cpm/membrane), and further incubated for 16hr at 65°C in a Hybaid mini oven. After 16hr hybridisation, the unbound DNA was removed by stringency washes. This consisted of 4 washes at RT for 5min each in 2XSSC and 0.1% SDS, solution followed by a 45min wash in 0.1% SSC and 0.1% SDS at 65°C. The membranes were removed from the hybridisation tubes, wrapped in Gladwrap individually, and exposed to Amersham Hyperfilm-MP in an autoradiography cassette for 2hr at -80°C. The film was immersed in Agfa G-150 developer for 5min, rinsed in flowing tap water and fixed in Agfa G-334 fixer for further 5min. The autoradiograph was then rinsed in water and air-dried.

3.2.9e. Isolating the DH5α colony clone containing the positively hybridised cosmid.

The autoradiogram was lined up with the colonies on the master plate and the positively hybridised colony was identified on the master plate. The colony was isolated, and purified and DNA extracted was analysed.

3.2.10. Complementation analysis.

3.2.10a. Triparental Mating

Triparental matings were used to transform the *Burkholderia* mutant strains with the complementing cosmid. The putative *gsp* cosmid was introduced into all mutants by triparental mating of the parents with a 'helper' strain, containing the plasmid pRK2013, which provided the conjugative and mobilising function in trans, to mobilise the pLAFR₃-based mobilizable cosmid. Eighteen hour old cultures of the donor *E. coli* DH5α strains carrying the cosmid, helper *E. coli* HB101 and recipients were washed twice with LB to remove the respective antibiotics, and 200μL aliquots of all the 3 were mixed in equal ratios (1:1:1). The resulting mixture was concentrated by centrifugation and resuspended in 100μL of fresh LB. The suspension was spotted onto a sterile 0.22 micron Millipore filter paper on a pre-warmed LB agar plate, and incubated for 16hr at

30°C. The bacteria were thereafter washed from the filter paper with 1mL of LB, harvested by centrifugation and resuspended in 400µL of LB. 200µL aliquots were spread on LB plates supplemented with antibiotics that would positively select the recipients with the cosmid, and incubated for 36-48hr at 30°C. Resultant transconjugants were selected from the plates, purified and checked for complementation.

3.2.10b. Complementation Assays.

Transconjugants were assayed for and compared to the mutants and the wild type to check for the:

(i) Capacity of forming cavities on mushrooms

The different test bacterial strains growing on LB plates supplemented with appropriate antibiotics were tooth picked on mushroom slices. The assay was set up duplicating all conditions stated in section 2.2.5.

(ii) Secretion of protease on the milk agar plates.

Milk agar plates were used to test the protease activity of the bacteria and using the assay described in section 2.2.7f.

(iii) Secretion of chitinase on chitin plates.

Chitin plates were used to test the ability of the complemented mutants to secrete chitinase using the assay mentioned in section 2.2.7e.

(iv) Morphological changes.

Morphological changes in the mutants in the presence of cosmid were noted by observing the transconjugants under a TEM. The bacterial samples were stained according to the method stated in section 2.2.7a. Stained bacteria were observed under bright field in a Hitachi H-600 electron microscope in the Mechanical Engineering Department of the University of Canterbury.

3.2.11. Analysis of secreted proteins.

3.2.11a. Preparation of protein samples

Bacterial cells were grown in mushroom extract medium (Appendix I) for 24hr, at the end of which bacterial cells were sedimented by centrifugation at 10,000rpm for 10min at 4°C. The supernatant was collected in a fresh sterile centrifugation tube, re-centrifuged and the supernatant was frozen immediately in liquid nitrogen. The frozen supernatant was dried in Edwards High Vacuum Centrifugal Freeze Dryer (model 30P.2. /822) available in the School of Biological Sciences. The samples were dried for 72hr with vacuum pressure set at 56 TORR, condenser temperature set at –50°C and the chamber temperature set at 30°C. The freeze dried samples were resuspended in 1/10th volume of 1 x MS solution (Appendix I) and purified by acetone precipitation, whereby, double the volume of ice-cold acetone was added to the sample, mixed thoroughly, incubated in –80°C for 30min, and the protein samples collected by centrifugation at 12,000rpm for 10min at 4°C. The supernatant was discarded and the pellet was resuspended in a half volume of 1 x MS solution, which made the final concentration factor 1:20.

3.2.11b. Estimation of the protein samples

Concentrations of protein in the solutions were measured using the Bio Rad Protein Assay Kit. This assay is based on Bradford's method to measure soluble proteins. Briefly, the assay consists of a colourimetric change in the dye, Coomassie[®] Brilliant Blue G-250, with varying concentrations of protein present in the solution. This dye has a shift in the absorbance maxima from 465nm to 595nm when protein residues bind to it, primarily aromatic and basic amino acids, such as arginine. Diluted protein samples (800µL) were incubated with the dye reagent (200µL) for 6min at room temperature and the absorbance of the coloured product was measured at 595nm in a Bio-Rad spectrophotometer. The concentration of the protein in the sample was measured by plotting the OD values against a standard curve drawn by plotting the OD₅₉₅ values of a standard BSA sample of known concentrations.

3.2.11c. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Sambrook *et al.* 1989)

Estimated quantities of secreted proteins were made up to 25 μ L volumes with sterile distilled water, to it, 25 μ L of 2 x Treatment Buffer (Appendix II) was added and the sample incubated for 90sec in boiling water to denature the proteins and reduce disulphide bonds in the protein solution. The samples were thereafter loaded on to a 4-20% mini gradient gel (commercially supplied by Gradipore) and electrophoresed for 1hr 30min at a constant voltage of 150 Volts in a Bio-rad mini PROTEIN™ II gel apparatus.

3.2.11d. Staining of the gels (Sambrook *et al.* 1989)

Protein profiles were visualised after being stained by silver nitrate solution. The solutions (Appendix II) were freshly prepared immediately before use. The gels were washed in wash solution I for 30min on a shaking platform, rinsed for 15min in distilled water, incubated in solution II for 30min, rinsed in distilled water briefly for 10min and then left washing in distilled water overnight at room temperature. The following day, the gels were stained for 10min in freshly prepared silver nitrate solution, washed three times for 5min each in distilled water and then the bands were developed by soaking in a developing solution. As soon as the bands appeared, the reaction was stopped by transferring the gel into a fixer. The gels were left overnight in the fixing solution to remove the background stains.

3.3. Results.

3.3.1. Cloning of transposon tagged genes in Cavity disease mutants.

Regions of BG164R chromosome flanking the transposon insertion sites were cloned in order to isolate the gene(s), interruptions that have rendered the mutant BG164R derivatives avirulent to Cavity disease formation. *SaII*-generated fragments of the chromosome were cloned using the vector pBluescript KS⁺ (Short *et al.*, 1988), which simplified sequencing because the multiple cloning site of the vector is flanked by opposing T7 and T3 promoters. The transposon (Fig 3.1.) has no internal *SaII* recognition sites, so all fragments should have DNA flanking the insertion. The kanamycin resistance cassette present in the transposon, mini-Tn5KmlacZ2, was used to select the clones, which carried genomic DNA from BG164R derivatives ranging in size from 8 to 11Kb (Table 3.2).

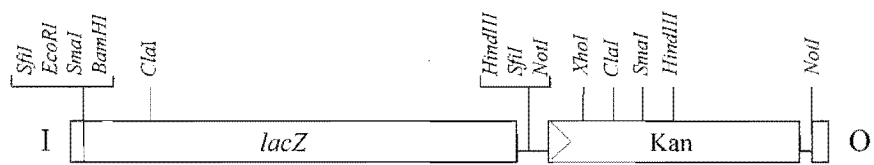


Figure 3.1: Schematic diagram of mini-Tn5 *lacZ*2 (deLorenzo *et al.*, 1990) [figure not to scale]

Mutant:	Plasmid construct:	Size of the recombinant plasmids (kb)	DH5α containing the clone
BG4-12	pSPRC12	11.4	CMC4-12
BG12-88	pSPRC88	8.1	CMC12-88
BG12-147	pSPRC147	8.2	CMC12-147
BG15-40	pSPRC40	8.3	CMC15-40
BG15-87	pSPRC87	8.9	CMC15-87
BG16-787	pSPRC787	8.3	CMC16-787

Table 3.2: Size of the recombinant plasmids isolated from shotgun cloning of no-cavity forming mutants of BG164R. The DH5α containing clones were named as ‘CMC’ designating cavity mutant clone, with the numbers in the suffix arising from the numbers of the respective mutants

3.3.2. Physical maps of the clones.

Restriction maps of the clones were generated using the enzymes *EcoRI*, *BamHI*, *HindIII* and *SaII*. Restriction maps of the different plasmid clones were drawn using Mac Vector software and are presented in Fig 3.2. Plasmid clones, pSPRC147 and pSPRC88 and pSPRC40 and pSPRC787 have identical maps and insertion points and are therefore most likely to be duplicates (Table 3.3.)

Plasmid Construct.	Size of the construct (Kb).	Size of genomic DNA cloned at	
		T3 promoter end	T7 promoter end
pSPRC12	11.4	1.5Kb	2.5Kb
pSPRC40	8.3	100bp	700bp
pSPRC147	8.2	500bp	350bp
pSPRC87	8.9	700bp	800bp
pSPRC787	8.3	100bp	800bp
pSPRC88	8.1	500bp	350bp

Table 3.3: Length of genomic DNA cloned on either side of the transposon insertion points in the recombinant plasmid clones.

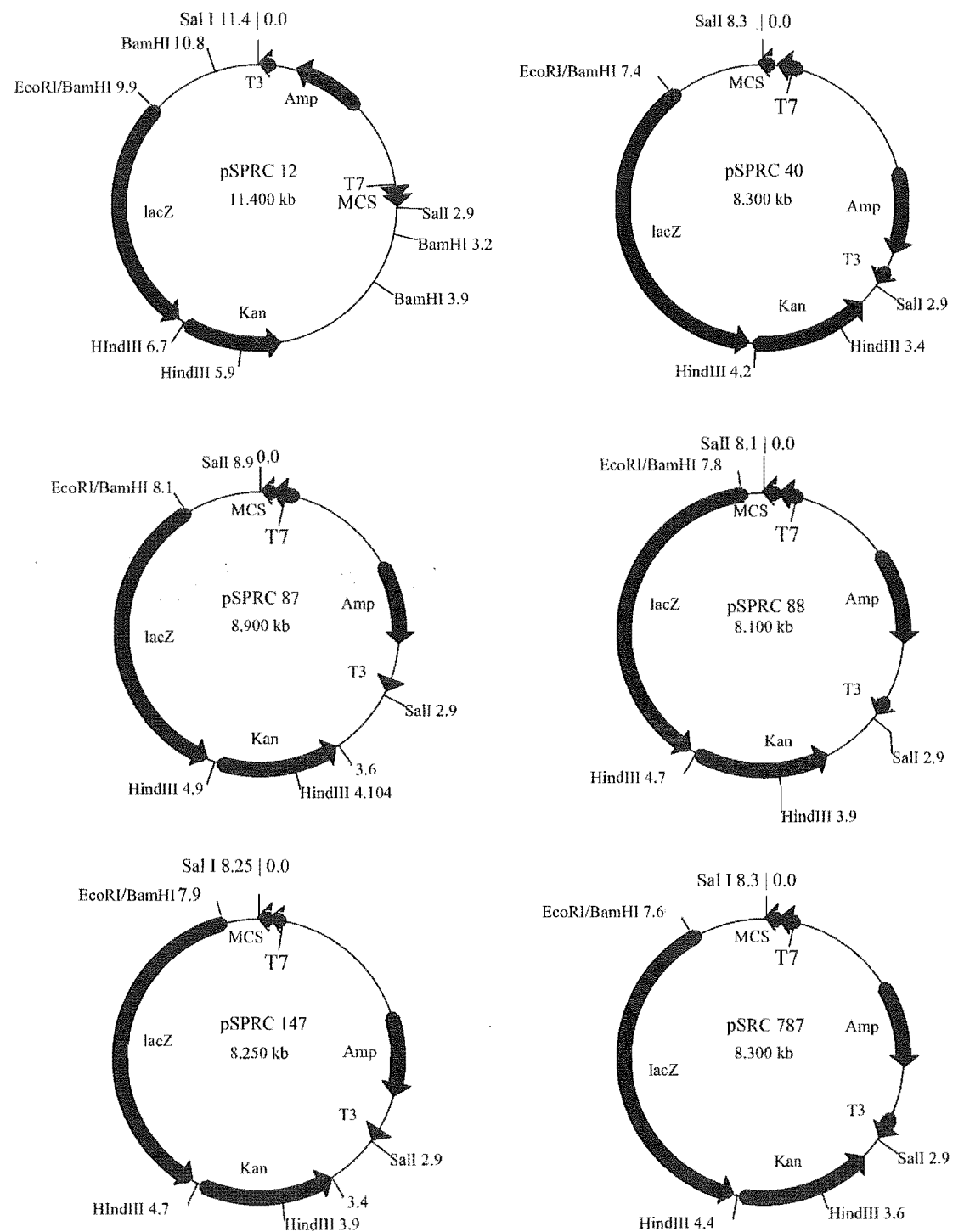


Figure 3.2.: Restriction maps of the plasmid clones arising from the no-cavity forming mutants of BG164R.

3.3.3. Number of transposon insertions in each mutant.

Southern hybridisation confirmed that each mutant had a single genomic insertion (Fig 3.3). The size of the *Sal*I genomic fragment containing the transposon was similar or identical among all the mutants, suggesting that each mutant was from an insertion in the same *Sal*I fragment.

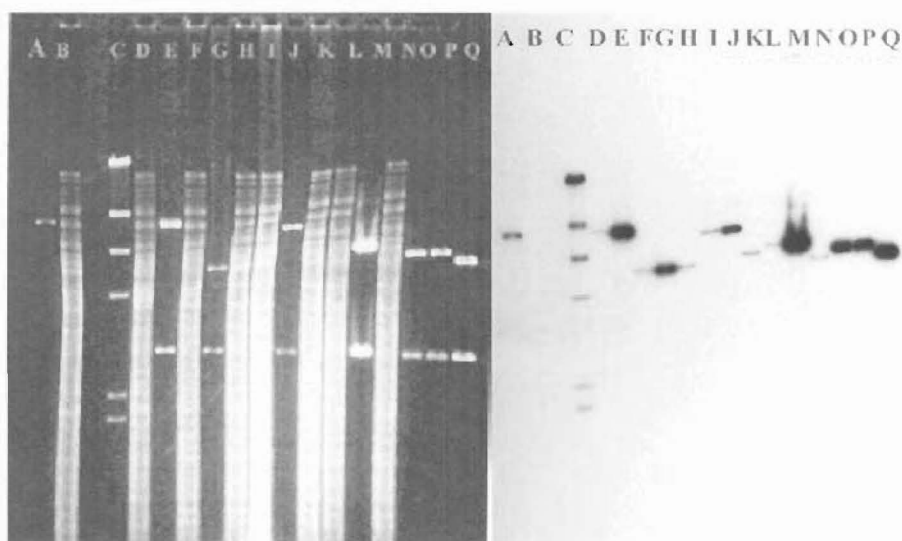


Figure 3.3: Southern blot analyses to ensure presence of a single transposon insertion in each of the cavity minus mutant of BG164R. The DNA gel in the left and the corresponding autoradiograph on the right. [A] pUT::mini-Tn5 (+ve control), [B] BG164R genomic DNA(-ve control), [C] λ HindIII marker, [D] BG4-12, [E] pSPRC12, [F] BG12-88, [G] pSPRC88, [H] BG12-147, [I] BG14-722, [J] pSPRC722, [K] BG15-40, [L] BG15-87, [M] pSPRC87, [N] BG16-787, [O] pSPRC787, [P] pSPRC40, [Q] pSPRC147.

The insertions were not in identical places within this *Sal*I genomic fragment, so each clone was sequenced to identify the possible open reading frames that may have been interrupted.

3.3.4. Sequence analysis of the mutant clones.

The sequence of the clones from the *SalI* genomic fragment are identical or highly similar at both the nucleotide and protein levels with the different 'gsp' genes of the 'General Secretory pathway' of two other species belonging to the genus *Burkholderia*, namely, *B. cepacia* strain KF1 with accession number AB050004.1 and *B. pseudomallei* strain 1026b with accession number AF110185 (Table 3.4). Detailed information about the statistical significance of the reported matches is presented in Table 3.5.

Mutant number	Plasmid clone	From T3 end of the vector			From T7 end of the vector.		
		Length of cloned DNA fragment	Length of sequence read out	Similar to gene	Length of cloned DNA fragment	Length of sequence read out	Similar to gene
BG4-12	pSPRC 12	1.5Kb	549bp	<i>gspE</i>	2.5Kb	709bp	<i>gspG, gspH</i>
BG12-88	pSPRC 88	500bp	266bp	<i>gspK</i>	350bp	99bp +Tn5	<i>gspK</i>
BG12-147	pSPRC 147	500bp	402bp +Tn5	<i>gspK</i>	350bp	59bp +Tn5	<i>gspK</i>
BG15-40	pSPRC 40	100bp	64bp +Tn5	<i>gspE</i>	700bp	429bp	<i>gspD</i>
BG15-87	pSPRC 87	700bp	255bp	<i>gspD</i>	800bp	375bp	<i>gspD</i>
BG16-787	pSPRC 787	100bp	64bp +Tn5	<i>gspE</i>	800bp	753bp +Tn5	<i>gspD, gspE</i>

Table 3.4: Results of similarity searching with sequences from the six 'no-cavity' causing mutant clones.

The cloned genomic DNA in pSPRC40 and pSPRC787 was identical (Tables 3.4 and 3.5), but the DNA of clones pSPRC88 and pSPRC147 was not. In the case of the latter two strains, the mutations are in the same locus of the chromosome, *gspK* gene, although the exact position of transposon insertion site is separated by 40 nucleotides, as evidenced from the T7 end sequence reads presented in Table 3.5.

The transposon inserted within 40 nucleotides of each other and into the same gene, in the mutants from which the clones pSPRC88 and 147 were derived, but into the same gene in the mutants from which the clones pSPRC40 and 787 were derived.

Plasmid Clone	Primer reads	Matches	<i>B. pseudomallei</i> (1026b) AF110185		<i>B. cepacia</i> (KF1) AB050004.1	
			% Identity	E-value	% Identity	E-value
pSPRC12	T3	<i>gspE</i>	85	e-115	87	e-136
	T7	<i>gspG</i> , <i>gspH</i>	89, 93	4e-76,3e-13	89,82	4e-76,1e-21
pSPRC40	T3	<i>gspE</i>	89	3e-06	95	1e-13
	T7	<i>gspD</i>	83	3e-50	92	7e-26
pSPRC88	T3	<i>gspK</i>	98	2e-18	81	3e-11
	T7	<i>gspK</i>	N/M	N/A	87	1e-10
pSPRC87	T3	<i>gspD</i>	83	9e-36	88	3e-63
	T7	<i>gspD</i>	84	1e-46	90	3e-65
pSPRC787	T3	<i>gspE</i>	89	4e-06	95	2e-13
	T7	<i>gspD</i> , <i>gspE</i>	85,80	3e-66,0.004	91,96	6e-32,0.069
pSPRC147	T3	<i>gspK</i>	98	3e-18	88	9e-07
	T7	<i>gspK</i>	N/M	N/A	86	1e-08

Table 3.5. Statistical significance of the matches with the two published sequences available in the database. N/M= no significant match found, N/A=Not available.

Further evidence that the insertions were sufficient to explain the mutant phenotype was provided by complementing mutations with DNA from a genomic library, described in section 3.3.7.

3.3.5. Construction of BG164R genomic library.

To ensure 99% probability of having a DNA sequence in the library, the minimum number of clones required to represent a genomic library consisting of 20kb genomic DNA fragments, was calculated on the basis of total genome size of *B. cepacia* and

B. pseudomallei genomes published by the Sanger institute. Although the calculations suggested the necessity of 1610 clones, it was decided to store 6000 clones to represent the complete genomic library, constructed according to the methods detailed in section 3.2.8.

3.3.6. Library probing and retrieving the wild type *gsp* gene containing cosmid(s).

Probing 3,200 library clones (double the number of clones required to find at least 1 copy of a single gene, as calculated), by colony hybridisation, initially led to the isolation of about 10 cosmid containing colonies. However, in repeat experiments, only one of the presumptive cosmids continually hybridised, thereby confirming its possible authenticity. The cosmid was named pCosGSP and stored for further analyses. The single cosmid isolated, pCosGSP, was found to have genomic insert of 23.4kb. The size of the GSP operon in the different species of *Burkholderia* varies from 9Kb to 14Kb (DeShazer *et al.*, 1999; Kimoto & Nakazawa, 2000), making it plausible that the pCosGSP held all the GSP genes.

3.3.7. Complementation of mutants with the cosmid.

To investigate the length of the *gsp* operon present in the cosmid and also to confirm the authenticity of the cosmid, pCosGSP was introduced by tri parental mating initially into two independent GSP mutants, namely BG4-12 and BG12-88 that had the transposons inserted in two different *gsp* genes- *gspF*, and *gspK*, respectively. The two specific mutants were initially chosen because, these two genes in the *gsp* clusters of closely related members of *Burkholderia* were present at two opposite ends of the operon. Thus, complementation of these two mutations by the same cosmid would possibly mean the presence of the whole *gsp* gene cluster of BG164R in the single isolated cosmid.

Four independent conjugations were set up for each of the two mutants. Twelve putative transconjugants for each mutant type were purified and tested in the mushroom bioassay (Fig. 3.4). Cosmid DNA from two representative transconjugants was subsequently re-

confirmed to have the same restriction map as the original cosmid prior to setting up of the mushroom bioassays to test for complementation.

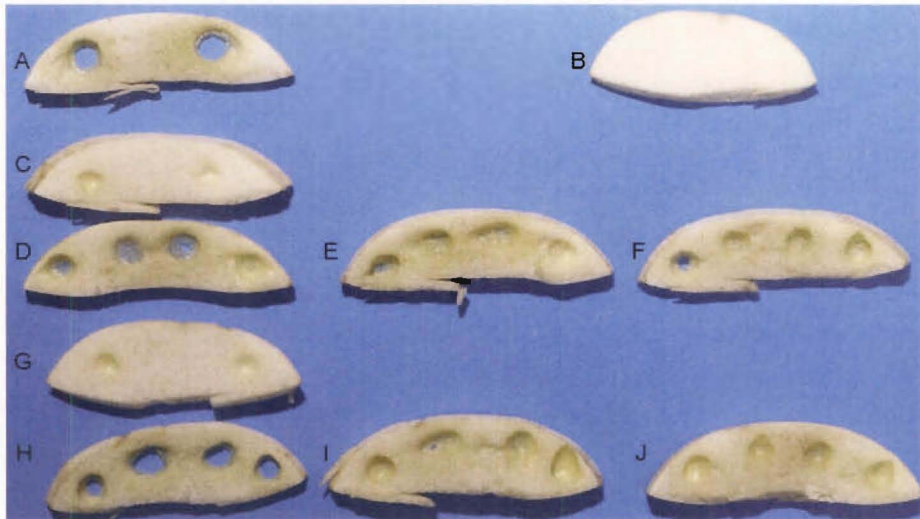


Figure 3.4: Mushroom slice assay to check for complementation. [A] inoculated with BG164R, [B] blank control, [C] BG4-12, [D-F] BG4-12Cos, [G] BG12-88, [H-J] BG12-88Cos.

Phenotypes of ability to degrade milk proteins (Figure 3.5), colloidal chitin (Figure 3.6) and the flagella number (Figure 3.7) in all the transconjugants were restored back to that observed in case of the wild type strains. However, the diameter of the zones of clearing for transconjugants on the milk agar plates was smaller compared to that of the wild type. This could be due to one of the two possibilities stated below:

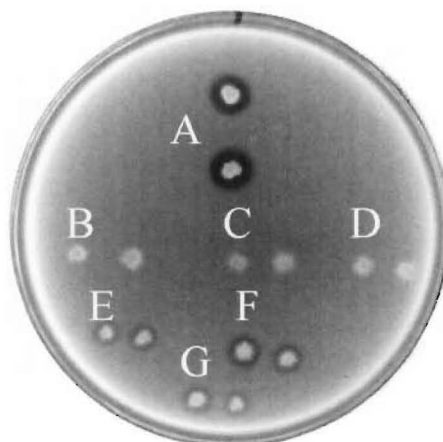


Figure 3.5A: Protease assay on milk agar plate to test for complementation. [A] BG164R, [B] BG4-12, [C] BG15-40, [D] BG12-88, [E] BG4-12Cos, [F] BG15-40Cos, [G] BG12-88Cos.

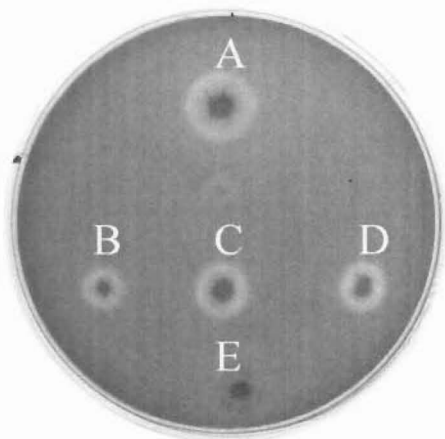


Figure 3.5B: Protease assay on milk agar plate supplemented with tetracycline to test for complementation. [A] control BG164R (pLAFR₃). [B] BG4-12Cos. [C] BG15-40Cos. [D] BG12-88Cos. [E] control BG4-12(pLAFR₃).

- (1) Partial complementation of secreted protease gene(s), which is/are present either upstream or downstream of the GSP genes of *Burkholderia* in the cosmid, and share a set of regulatory genes with the GSP operon.
- (2) The presence of more than one protease gene involved in casein degradation on the milk agar plates, of which only one is being complemented by the cosmid.

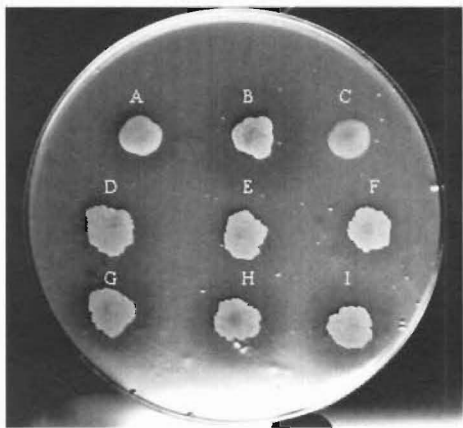


Fig 3.6: Chitinase assay to test for complementation. [A] *Serratia entomophila* A₁MO₂ control, [B] BG164R, [C] *P.cepacia* B111, negative control [D] BG4-12, [E] BG15-40, [F] BG12-88, [G] BG4-12Cos, [H] BG15-40Cos, [I] BG12-88Cos.

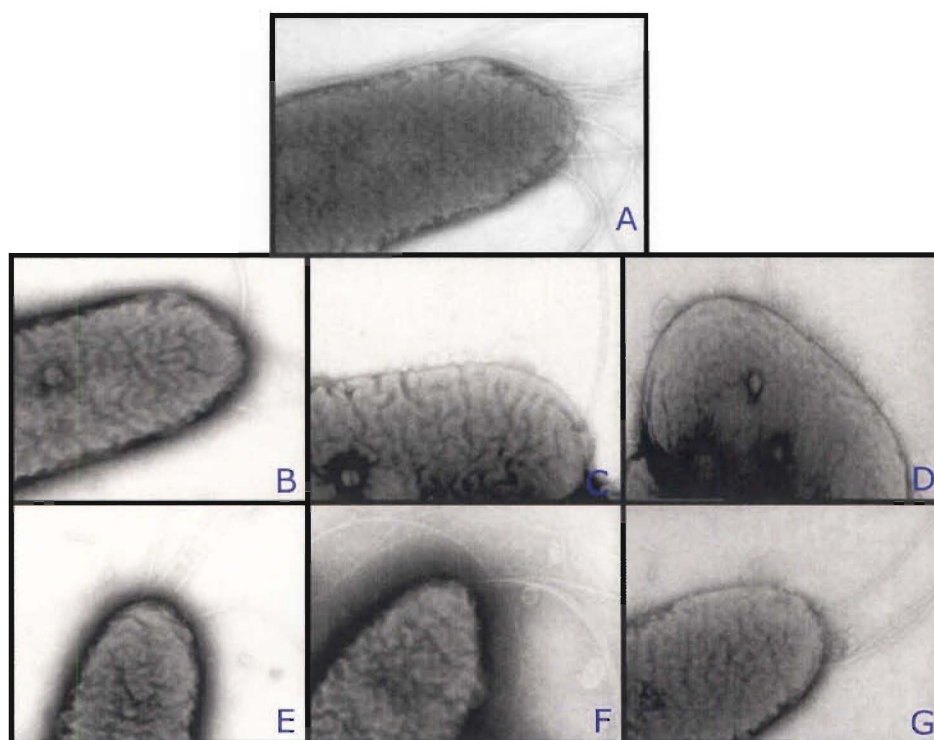


Fig 3.7: Transmission electron microscopic observations to test for complementation. Observations of wild type, mutants and the complemented mutants. [A] BG164R, [B] BG4-12, [C] BG15-40, [D] BG12-88, [E] BG4-12Cos, [F] BG15-40Cos, [G] BG12-88Cos.

The reduced capacity to degrade colloidal chitin in mutants was restored back to the normal in the presence of the cosmid. Restoration of flagellar number was observed for all the transconjugants harbouring the GSP cosmid and can be morphologically compared to the wild type. About 75% of the bacteria in the observation frame exhibited the above-mentioned phenotype. The difference in phenotype noticed in the remaining 25% could be attributed to the method of preparation of the TEM grid.

3.3.8. Secretion profiles of the wild type and a *gsp* mutant.

Differences in the profiles of secreted proteins were expected to be visible in the culture supernatants, since the mutants isolated were all secretory mutants. To test this hypothesis, supernatant proteins were extracted from wild type and a representative GSP mutant (BG15-40) and compared on SDS PAGE (Figure 3.8). All the protein samples were diluted to uniform concentration of 3µg protein /10µL of sample extract

diluted in 1x MS and the size fractionated proteins were stained with silver nitrate solution.



Figure.3.8: Lane [A] Protein gel marker. Sizes in kD marked along the side. [B] blank row. [C] blank LB media extract. [D] extract of BG164R supernatant growing in LB media. [E] extract of BG15-40 growing in LB media. [F] blank lane. [G] blank MEM extract. [H] extract of BG164R supernatant growing in MEM. [I] extract of BG15-40 supernatant growing in MEM. [J] blank lane. [*] diagrammatic representation of the distinct bands that appear in lane H.

Five distinct bands observed in lane (H) loaded with extracts from BG164R grown in mushroom extract media. All five bands were absent in the corresponding lane (I) loaded with extracts from the mutant bacteria grown in the same media. Interestingly, there are some common bands observed in the extracts of wild type grown in LB media (lane D). All six extracts were spotted on milk agar plates. Protease activity was evident in BG164R culture supernatants extracted from both the media (Figure 3.9). This could possibly indicate that proteases in the supernatants represent the common bands observed in case of both the media extracts. Notably the three common bands appearing in the lanes D and H do not have any counterpart in the lanes in which their respective mutant extracts from both the media are loaded (lanes E and I). Also, the upper four prominent bands appearing in the BG164R ME media lane does appear to be present very faintly in the BG164R LB extract lane, indicating minimal levels of expression of the proteins represented by the three upper bands in the absence of mushroom specific compounds in the culture media. These differences in the banding patterns have been consistently observed in all the 3 repeat gels run to ensure the authenticity of the results.

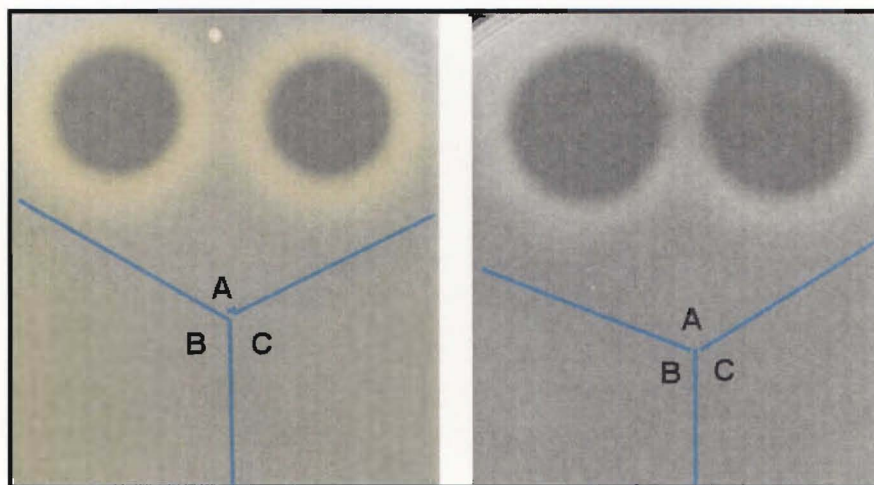


Figure 3.9: [Left] Protein extract from LB media spotted on Milk Agar plate. [Right] Protein extract from MEM spotted on Milk Agar plate. [A] Quadrant containing extract from wild type BG164R supernatants. [B] Quadrant containing extract from media (control). [C] Quadrant containing extract from mutant BG15-40 supernatants.

3.3.9. Screening of mutants on Milk agar plates.

In an attempt to find one or more structural gene(s) for protease which could serve as one of the possible pathogenic factors associated in the expression of Cavity disease, a second mutagenesis screen was carried out and the transconjugants were screened initially on milk agar plates. Protease deficient strains were tested on mushroom slices to check for the ability to form cavity.

Of the 3,000 transconjugants screened on milk agar plates two mini-Tn5Km/*lacZ*2 mutants of BG164R were selected as protease minus on 1% milk agar plates. The two isolated mutants were tested on mushroom slices for the capacity of expressing Cavity disease. Interestingly, both the mutants lost the capacity of cavity formation on mushrooms. Since the main objective of this experiment was to identify protease gene(s), products of which could be linked to formation of cavity disease, shotgun cloning of the genomic region adjacent to the transposon insertion point into the sequencing vector pBluescript KS⁺ was carried out. The two clones isolated from the respective mutants, namely pBGProt-123 and pBGProt-125, had insertions on 6.6kb and 8.3kb respectively. Sequence analyses of the regions adjacent to the site of insertion of

transposon revealed the presence of mutations in two different proteins of general secretory pathway, not isolated in the previous screen. The clone pBGProt-123 had a mutation in the *gspL* gene while the clone pBGProt-125 had a mutated *gspF* gene.

3.4. Discussion.

Identification of the genes of *B. gladioli* pv. *agaricicola*, BG164R involved in the expression of Cavity disease on button mushrooms was the primary objective of the chapter. Cloning followed by sequence analysis of the genomic DNA adjacent to the transposon tagged genes in the mutant clones suggested clustering of mutations in the *gsp* operon of BG164R. Complementation of three *gsp* mutants with the cosmid pCosGSP, restored the wild type phenotypes in the mutants, thereby establishing involvement of GSP in the expression of Cavity disease by BG164R on mushrooms. Cloning of the mutants with no-cavity phenotype in case of BG164R was restricted to *SalI* as this was the only enzyme, which did not have any site on the transposon. Sequence analysis of the mutated genes led to the identification of *gspE*, *gspK*, *gspD*, *gspF* and *gspL* genes in the *gsp* operon of BG164R. However, because *SalI* had too many sites along the operon, the size of the fragments of genomic DNA present on either side of the transposon was not sufficient to analyse the complete ORFs of the genes mutated.

Screening of protease mutants on milk agar plates was attempted in an effort to identify the protease gene(s), production of which appeared to be important for the formation of Cavity disease. Sequence analysis of the two mutants isolated in the process also led to identification of genes of the GSP. Similar experiments conducted by DeShazer *et al.* (1999) and Nakazawa *et al.* (1996) have been reported in which all the mutants isolated from milk agar plates were clustered in the GSP operon. An argument that crops up in this case is whether the number of transconjugants screened to isolate protease mutants was sufficient enough to isolate unique mutants. DeShazer *et al.* screened 15,000 transconjugants and isolated 29 mutants all of which were clustered in the GSP operon. Nakazawa *et al.* screened 5000 transconjugants and isolated 14 mutants, of which, only one mutant had a mutation in the *dsbB* gene that is required for the proper folding of the

proteins that are secreted by the GSP. Thus, the inability to identify structural genes of the protein-degrading enzyme was not surprising.

None of the mutants isolated had mutations in any other genes, products of which were directly responsible for the expression of disease symptoms in mushrooms. It is tempting to suggest that Cavity disease is a combined manifestation of more than one factors secreted through the Type II protein secretory system present in BG164R. The secreted proteins are capable of causing disease symptoms only when present together on the substrate. Hence, to see a distinct no-cavity phenotype all the factors have to be absent, which is possible only when a common pathway involved in the secretion of them are blocked. When the products are produced individually the effects are either not detectable in the assay system used to screen for mutants, or they do not show any noticeable difference of the symptoms. Gill and Tsuneda (1997) in their study on the symptom development in the different types of mushrooms have also proposed a similar hypothesis. They suggested that chitinase and β -glucanases of *B. gladioli* pv. *agaricicola*, BG164R, act together to cause Cavity disease, since activity of these two enzymes were detectable in *in vitro* plate assays. Differences in the secreted protein profiles were clearly visible when the wild type and mutants were grown in mushroom extract media and not in LB. However, the culture supernatants of mushroom extract did not show any activity on chitinase plates or on the mushroom tissues although it retained the capacity to degrade milk protein. The mutants, when complemented with the cosmid, regain the capacity to degrade casein on milk agar plates, degrades colloidal chitinase to a similar extent as that of the wild type and degrade mushroom tissue. This re-enforces the hypothesis that more than one factor is necessary for the successful expression of Cavity disease.

Gill and Tsuneda (1997) proposed that the pathogen secretes/produces mushroom mycelia degrading components on induction, but, they did not have any concrete evidence for such a phenomenon being involved in the expression of Cavity disease *in vivo*. To assess the possibility of isolation of the active cavity causing compound(s) being produced only on induction, a bioassay was initially set up to check for the spontaneous production of any active compound(s) into the growth media. BG164R was grown on M63 agar media, LB agar, PDA plates and MEM agar media for three days;

plugs of agar were taken from the plate and placed on mushroom slices. The assay was incubated at 25°C overnight. Plugs of agar containing fresh media from a plate on which BG164R was not grown was taken as the control for each set of test media. A slight indent on the surface of the mushroom tissues could be observed with plugs of agar taken from the PDA plates and MEM agar media on which BG164R cells were grown, although this was not as devastating as the control slices of mushrooms inoculated with live BG164R cells. An interpretation for the observation of such a phenomenon could be a limitation in the amount of active compounds being present in the plug of agar, which was sufficient to cause the indent, but not enough to form the cavity. However, an interesting fact in this case was that no such indent was observed when agar plugs were taken from minimal agar or LB plates. The assay was repeated three times with the same results being noted in each case. The result either indicates the association of specificity of media with the production of active compounds, or indicates that the activity could be detected when bacteria were grown in a media with acidic pH. The former two media had a neutral pH around 7 while the latter two had a pH of 5.1, besides containing plant extracts.

Gill and Tsuneda (1997) have also reported the observation of such indents on mushroom tissues inoculated with a cell free culture supernatant extracted from BG164R cells growing in nutrient broth. Notably, nutrient broth is not a plant extract with a pH of 7.4. Gill reports shrivelling of tissue at the site of inoculation in spite of not having any signs of degradation of hyphal cell walls, from electron microscopic observations with pieces of tissue taken from the zones of depression. Mushroom slices inoculated with mutants also show such shrivelling of mycelia. If Cavity disease could be associated with the production of more than one compound, formation of such indents could be attributed to the production of some, but not all of, the compounds needed for the manifestation of the disease.

Mueller-Cajar (2001), working in a parallel project aimed to isolate the antifungal compound(s) produced by BG164R, reported antifungal activity from plugs of agar taken from PDA plates. He successfully extracted the active antifungal compound from PDA agar with acetone (Muller-Cajar, 2001). The acetone extract, which showed active antifungal property, was tested for its ability of cavity formation on mushroom slices.

No such activity was observed on mushroom slices. Indent formation on mushroom slices was also not observed in this case, and was rationalised as the compound being inactivated or denatured in the process of acetone extraction. Thus, although the active principle involved in the disease formation could not be extracted, the co-relation between general secretory pathway and Cavity disease formation has been established by the data presented in this chapter.

The general secretory pathway or Type II protein secretion in bacteria forms a terminal branch of secretion of toxins and proteins across the outer membrane in Gram-negative bacteria (Pugsley *et al.*, 1997b). The type II secretion system is generally associated with the secretion of more than one structurally diverse gene products and the only example where type II secretion machinery is associated with the production of one single exo-product is in *Klebsiella oxytoca* which secretes pullulanase (Pugsley *et al.*, 1997a). Incidentally, this is also the sole example where the structural genes for pullulanase production are clustered with the genes involved in the secretion. In other systems, where more than one protein is secreted by the *gsp* system in bacteria (Wandersman, 1996), there still appears to be single secretion machinery responsible for the secretion of all the polypeptides. The mechanisms by which all these different proteins are identified by the same secretion apparatus is as yet unknown.

The proteins forming the secretion apparatus of general secretory pathway in members of the genus *Burkholderia* have been reported to be clustered in a single operon, and are responsible for the secretion of protease, lipase and phospholipase C (DeShazer *et al.*, 1999; Kimoto & Nakazawa, 2000). In the three *gsp* gene clusters studied in the different members of *Burkholderia*, the structural genes responsible for the production of the exo-enzymes have not been found to be associated with the genes forming the secretion apparatus and the bacteria has been studied as a human pathogen causing nosocomial infections in immuno-compromised patients. *Burkholderia gladioli* pv *agaricola*, BG164R, is the first reported pathogen of mushrooms, and is involved in the formation of a massive tissue degradative disease in mushrooms. This is hence the first report on the association of the involvement of general secretory pathway of a pathogen to the expression of symptoms in case of a mushroom disease.

3.5. Concluding Remarks.

Mutagenesis screenings of BG164R in association to Cavity disease lead to the identification of genes coding for GspE, GspK, GspD, GspF and the GspL counterparts of the type II secretion apparatus of *B. gladioli* pv.*agaricola*. Further genetic analysis of pCosGSP to genetically define the borders of the *gsp* gene cluster present in cosmid pCosGSP forms the main theme of the study presented as chapter four.

Chapter IV

Analysis of pCosGSP.

4.1. Introduction.

The construct pCosGSP is a cosmid that contains the putative *gsp* (General Secretory Pathway) gene cluster of *B. gladioli* pv. *agaricicola* strain BG164R, the causal organism of Cavity disease in mushrooms. The no-cavity mutants isolated in this study harbour mutations in different genes of the *gsp* operon of *B. gladioli* pv. *agaricicola*. The genes were identified by sequence analysis of clones containing genomic DNA adjacent to the transposon-tagged mutated genes. Subsequently, a genomic library of BG164R was constructed in the cosmid pLAFR₃. Screening of this library led to the isolation of pCosGSP, which, as described in section 3.3.7, complements mutations in three separate genes along the *gsp* operon. This was observed by restoration of wild type phenotypes including cavity formation (on mushroom slices), flagella number and protease secretion in *in vitro* assays.

The GSP operon of three different members belonging to the genus *Burkholderia* has been studied in association to pathogenesis (Mitsuko & Nakazawa, 1996; Nakazawa & Mitsuko, 1996; DeShazer *et al.*, 1999). The complete GSP operon has been identified and mapped in *B. pseudomallei* (DeShazer *et al.*, 1999), and, as mentioned in section 3.3.4, has significant sequence similarities with the different proteins constituting the *gsp* operon of BG164R. All the strains studied and reported previously were clinical isolates and an underlying feature connecting all these studies was the secretion of extracellular protease, some of which have been directly linked to the pathogenesis of the respective strain.

DeShazer *et al.* (1999) in their study on the causative agent of melioidosis, *B. pseudomallei* 1026b, screened transposon mutants with an aim to identify the genes

involved in the secretion of protease, lipase and phospholipase C. The ultimate objective of the study was to correlate the effects of these exo products to pathogenesis. The 29 mutants selected initially did not secrete protease. In each case, they also appeared to be lipase and phospholipase C deficient. This phenotypic linkage indicated that the mutation was in a common secretory pathway. Sequencing of the mutated genes revealed that the mutations were in different genes of the general secretory pathway. The two boundaries of the *gsp* operon were identified by constructing mutants specifically in the *orfC* and the *orfD* genes present in the left and right ends of the gene cluster respectively, which did not have any effect on the secretion. A centrally located gene, *gspC*, transcribed in the opposite direction to the other genes in the operon, was also identified. The sequence of 20kb genomic DNA of *Burkholderia pseudomallei* 1026b encompassing the *gsp* genes was published in the database. When the relative virulence of the wild type and a *gspD* mutant strain was compared in the Syrian hamster model, they did not find any major difference in the capacity of infection, thereby suggesting that the secreted protease, lipase and phospholipaseC played a minor role in the pathogenesis of the strain (DeShazer *et al.*, 1999).

Mitsuko and Nakazawa (Mitsuko & Nakazawa, 1996; Nakazawa & Mitsuko, 1996) in their analyses on the different clinical strains of *B. cepacia* screened transposon mutants of strain KF1 to isolate protease mutants. Thirteen out of the 14 selected protease mutants did not secrete lipase. A protease reduced and lipase deficient phenotype (KF1008) mapped to a mutation in the *gspF* gene, while the mutation in the protease deficient and lipase-producing mutant (KF1007) was identified to be in the *dsbB* gene. Interestingly, the *dsbB* mutant was nonmotile, and aflagellate, and these phenotypes could be restored by complementation of the mutant with a plasmid containing the *dsbB* gene. The *dsbB* gene in *E. coli* is responsible for the formation of disulfide bonds in periplasmic proteins. On the basis of the results and correlation of the steps involved in the secretion of proteins by GSP in other studied models, *dsbB*-mediated efficient protein folding by *B. cepacia* KF1 was proposed to be involved in protein secretion by GSP. However, the folding of prelipase is dependent on a molecular chaperone Lim, encoded by the gene *limA*. Hence, the *dsbB* mutant did not show any difference in the secretion of lipase. In a second publication by the same authors, anti KF1 protease antibody was used to identify the size of the proteases secreted. This revealed the

presence of two KF1 protease bands, of which the smaller band was similar to the purified protease of KF1. This led the authors to speculate that the protease precursor was 43 kDa which, when processed and secreted, was 37kDa in size. The above prediction was supported by the fact that the 43kDa band was also present in the *dsbB* mutant. The observation also emphasises the notion that *dsbB* is essential in the secretion of protease in *B. cepacia* KF1, which in turn is secreted by the general secretory pathway.

Corbett *et al.* (2003) have correlated virulence to the production of a zinc metalloprotease, which also appeared to be secreted (Corbett *et al.*, 2003). The metalloprotease had a presecretory protein and the mature proteins of similar sizes to that suggested by Mitsuko and Nakazawa (1996). Corbett *et al.* (2003) isolated the gene coding for the zinc metalloprotease (*zmpA*) from *B.cepacia* K56-2 and Pc715j. Mutations in the *zmpA* gene made the strains avirulent in rat infections. The primary amino acid sequence of the metalloprotease suggested the presence of a signal sequence thereby *sec*-dependent transport of the protease across the inner membrane of the strain and consequently GSP-dependent transport across the outer membrane was proposed. Thus, studies mentioned above have successfully correlated the proteins secreted by GSP to the pathogenicity in animal models. To the best of my knowledge, there has not been any such association described in the case of a mushroom disease.

The main focus of this chapter is the analysis of the *gsp* cosmid, pCosGSP, in order to physically locate the genes of *gsp* operon on the cosmid and identify whether the *gsp* operon in this specific cosmid is associated with any other structural genes. In the last section of this chapter, results from a second mutagenesis screen are described, including cloning and identification of the mutated genes. This mutagenesis screening was undertaken as a repeat attempt to identify more gene(s) involved in the expression of Cavity disease.

4.2. Material and Methods.

4.2.1. Bacterial strains and plasmids created or used.

Strains and plasmids	Genotype or description	References
<i>Burkholderia gladioli</i> pv. <i>agaricicola</i> .		
BG164R	wildtype, cav+, Af+, Rif ^R , Prot+	This study
BG4-12	BG164R <i>gspF</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG12-88	BG164R <i>gspK</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BG15-40	BG164R <i>gspE</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BGII-105	BG164R <i>gspE</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-,Af+, Prot-	This study
BGII-106	BG164R <i>gspK</i> ::mini-Tn5KmlacZ2,Rif ^R ,Km ^R , cav-, Af+, Prot-	This study
BGII-107	BG164R <i>pks</i> ::mini-Tn5Km lacZ2,Rif ^R ,Km ^R , cav-, Af+, Prot-	This study
BG4-12Cos	BG4-12 complemented with cosmid pCosGSP, Rif ^R , Kan ^R , Tet ^R ,cav ⁺	This study
BG12-88Cos	BG12-88 complemented with cosmid pCosGSP, Rif ^R , Kan ^R , Tet ^R ,cav ⁺	This study
BG15-40Cos	BG15-40 complemented with cosmid pCosGSP, Rif ^R , Kan ^R , Tet ^R ,cav ⁺	This study
BG4-12ME	BG4-12 complemented with pGSP ME H-H16.7, Rif ^R , Gent ^R , Kan ^R ,cav ⁻	This study

Eschericia coli

DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 thi-1 relA1 recA1</i>	Hanahan 1983
HB101	<i>supE44 hsdS20 (r_B-m_B-) recA13 ara-14 rspL20 proA2 lacY1 galK2 xyl-5 myl-1</i>	Boyer 1969

Pseudomonas fluorescence

PRC121	Wild type mushroom isolate, Prot-	This study
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Plasmids and constructs

pSPRC105	4.4kb <i>SaI</i> I fragment containing <i>gspF</i> ::mini-Tn5 <i>KmlacZ2</i> from BGII-105 in pBluescript KS+, Ap ^R , Kan ^R .	This study
pSPRC106	4.7kb <i>SaI</i> I fragment containing <i>gspK</i> ::mini-Tn5 <i>KmlacZ2</i> from BGII-106 in pBluescript KS+, Ap ^R , Kan ^R .	This study
pSPRC107	7kb <i>SaI</i> I fragment containing <i>pks</i> ::mini-Tn5 <i>KmlacZ2</i> from BGII-107 in pBluescript KS+, Ap ^R , Kan ^R .	This study
pCosGSP	23.4kb <i>Sau3A</i> I partial fragment containing GSP gene cluster from BG164R into pLAFR ₃ .	This study
pGSP ME H-H16.7	16.7kb <i>Hind</i> III subclone from pCosGSP in pME6001, Gent ^R .	This study
pGSP ME H-H2	2kb <i>Hind</i> III subclone from pCosGSP in pME6001, Gent ^R .	This study
pGSP ME H-E1.7	1.7kb <i>Hind</i> III-EcoRI subclone from pCosGSP, in pME6001, Gent ^R .	This study
pGSP KS B-B2.2	2.2kb <i>Bam</i> HI subclone from pCosGSP in pBluescriptKS+, Ap ^R .	This study
pGSP KS B-B7.2	7.2kb <i>Bam</i> HI subclone from pCosGSP in pBluescriptKS+, Ap ^R .	This study
pGSP LA H-H2	2kb <i>Hind</i> III subclone from pGSP ME H-H2 in pLAFR ₃ , Tet ^R .	This study

pROBE	1.5kb <i>NotI</i> fragment containing the kanamycin gene from mini-Tn5KmlacZ2blunt end cloned into the <i>EcoRV</i> site of pBluescriptKS-; Ap ^R	S.R.Giddens
Vectors.		
pBluescript (KS,M13+)	ColE1 <i>ori lacZα</i> /KS polylinker, T3/T7,Ap ^R	Stratagene
pLAFR3	pRK290 derivative;RP4(IncP-1) <i>ori</i> , λ cos, pUC9 multicloning site and <i>lacZα</i> ,Tc ^R (22kb)	Staskawicz <i>et al.</i> 1987
pME6001	pBBR1MCS derivative; ColE1 <i>ori lacZα</i> /multiple cloning site polylinker, mob,rep; Tc ^R (7.2kb)	Stephan Heeb

Table4.1. List of strains and constructs used in the experiments described in chapter 4.

4.2.2. Media, reagents and antibiotics.

All media and reagents used in this study were prepared as described in Appendices I and II. Concentrations of the antibiotics used to maintain the selective pressure on the strains or the strains containing plasmid or cosmid constructs are as described in Appendix I, unless otherwise stated.

4.2.3. Bacteriological methods and mushroom bioassay system.

The bacteriological methods and the cultural conditions used were as stated in section 2.2.3. All mushroom bioassays were done following the method described in section 2.2.5.

4.2.4. DNA manipulation techniques.

The DNA manipulation and cloning techniques, southern hybridisation, electro transformation and tripartite mating experiments were done following the techniques

described in section 3.2, unless otherwise stated. Mini-Tn5 transposon mutagenesis experiments were done according to the technique standardised and described in section 2.2.6.

4.2.5. Cosmid stability and mutant rescue assays.

4.2.5a. In liquid media.

Transconjugants were cultured without antibiotic, starting from 1:100 dilution of a saturated culture growing overnight for 18hr as the initial inoculum. After every 24hr, for 3 days, 100µL aliquots were aseptically removed from the cultures, serially diluted to a factor of 10^6 , and two sets of 100µL aliquots from each were spread on LB plates and incubated for 24hr. A titre of viable colony forming units was calculated from the dilution series. A grid of 200 colonies from each plate (400 for each transconjugant) was made on a master LB+ rifampicin (Rif) plate. Tetracycline (Tet) and kanamycin (Km) resistance markers were checked by transferring the colonies to LB+Rif+Tet plates and LB+Rif+Km, respectively, to score for transconjugants that have lost the cosmid or the transposon. Colonies that were sensitive to both kanamycin and tetracycline were selected as putative wild type strains re created by an event of double crossover or allelic exchange. Such putative wild type strains were later verified by a Southern blot analysis to confirm the loss of the transposon.

4.2.5b. On mushroom slices.

Individual transconjugant colonies growing under appropriate antibiotic selection were tooth picked on to thick mushroom slices and the mushroom assays were set up according to the standardised conditions. After sixteen hours of incubation, a sterile loop was used to transfer bacteria from the mushroom slice to 100µL of LB and vortexed to make a uniform suspension. This bacterial suspension was thereafter serially diluted by a factor of 10^4 and 100µL of the dilution of cells were spread on a rifampicin supplemented plate, to avoid contamination by the bacteria coming from the mushroom pieces, and incubated at 30°C for 24hr. The total population of mutants surviving on the mushroom pieces were scored and a representative population of it was replica tooth picked on all selection plates mentioned in section 4.2.5a. to check the true

population of transconjugants surviving and also to calculate the rate of recombination. The assay was repeated twice; in the first set-up, 400 colonies arising per transconjugant of each mutant were tested on the selection plates, while in the second set-up, 300 colonies per transconjugant were tested. Results presented are average percentages arising from screening 700 colonies in each set of transconjugant.

4.2.6. Verification of strains rescued by recombination.

Initially, mushroom assays were set up to test the capacity of cavity formation by the putative rescued mutants from all the three mutants used in the experiment. The recreated strains were purified on selective plates, genomic DNA was prepared and a southern blot analysis was done according to the method stated in section 3.2.6. with a probe constructed from the 1.5kb *NotI* fragment of the kanamycin cassette from the transposon mini-Tn5KmlacZ2 (pROBE) to confirm the results arising from mutant rescue assays.

4.3. Results.

4.3.1. Restriction analysis of pCos GSP-BG

The exact sizes of the expected *SaII* fragments within the *gsp* gene cluster of BG164R were theoretically calculated from the size of the mutant clones (Table 4.2.). The total size of the genomic DNA present in the cosmid was calculated from the fragments arising from *EcoRI* and *HindIII* double digest of the cosmid (Figure 4.1.), as these sites were present on either side of *BamHI* site in the MCS of pLAFR₃ exploited to construct the library.

Plasmid construct	Total size of the construct.	Size of genomic DNA clones		Expected band sizes on the gel	
		T3 end	T7 end	From <i>Sal</i> I digests	From <i>Bam</i> HI digests
pSPRC12	11.4kb	1.5kb	2.5kb	4kb	2.2kb+700bp
pSPRC40	8.3kb	100bp	700bp	800bp	NA
pSPRC147	8.2kb	500bp	350bp	850bp	NA
pSPRC87	8.9kb	700bp	800bp	1.5kb	NA

Table 4.2: Size of expected DNA fragments arising from a *gsp* cosmid, isolated from the genomic library. NA= No restriction site present in the genomic DNA of the clones.

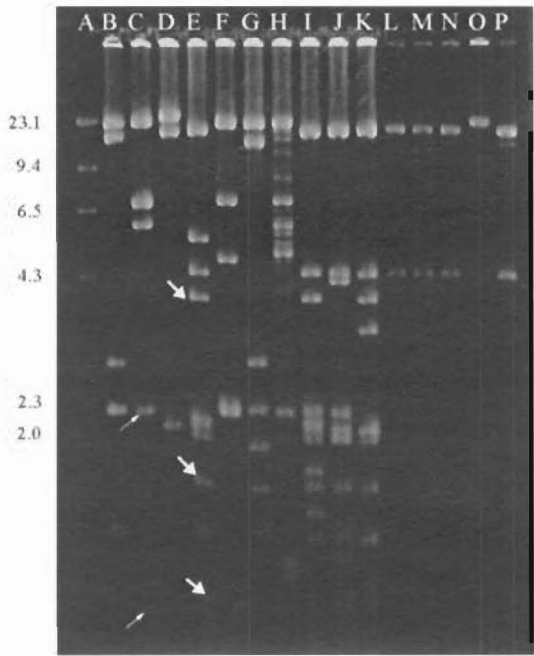


Figure 4.1:Restriction analysis of p CosGSP-BG. A= λ /HindIII marker DNA. Lanes B-K= pCosGSP digested with different enzymes. B=*Eco*RI, C=*Bam*HI, D=*Hind*III, E=*Sal*I, F=*Eco*RI+*Bam*HI, G=*Eco*RI+*Hind*III, H=*Hind*III+*Bam*HI, I=*Eco*RI+*Sal*I, J=*Bam*HI+*Sal*I, K=*Hind*III+*Sal*I, Lanes L-P=pLAFR₃ digested with different enzymes. L= *Sal*I, M=*Eco*RI+*Sal*I, N=*Bam*HI+*Sal*I, O=*Bam*HI, P=*Hind*III+*Sal*I. Arrows pointing to bands of interest in lanes C & E.

The predicted bands (indicated by arrows in Fig 4.1) were confirmed. Smaller arrows pointing upward in lane C indicates the expected internal *Bam*HI bands that were

mapped in the clone pSPRC12. In lane E, the approximate sizes of the *Sa*II fragments as calculated are also present and marked with bold arrows pointing downwards. When compared to lane J, where the cosmid is digested with both *Sa*II and *Bam*HI, the 4kb *Sa*II band disappears, with the appearance of the 2.2kb and the 700bp internal *Bam*HI bands. Adding up the sizes of the six bands evident from the gel in lane G, the cosmid has an insert of 23.4kb of genomic DNA.

The number of restriction sites in the 23.4kb genomic DNA could be determined from the restriction profile, however, a physical map of the cosmid could not be constructed solely based on this data. Pugsley *et al.* (1997b) said that the relative order of the genes coding for the different components of the type II secretion machinery are generally the same along the chromosome of all the bacteria studied so far. If true, then a physical map of the *gsp* gene cluster in the BG164R can be provisionally drawn based on the published *gsp* gene clusters of *Burkholderia* spp (DeShazer *et al.*, 1999; Kimoto & Nakazawa, 2000).

4.3.2. Orientation *gsp* genes in BG164R.

The relative order of the *gsp* genes in the strain *B. cepacia* strain KF1 (Figure 4.2) (Kimoto & Nakazawa, 2000) was used as a guide to assemble the *Sa*II fragments of BG164R cloned and sequenced from the mutants. Initially, with the information available in the database (Kimoto & Nakazawa, 2000) a representation of the order in which the *gsp* genes were arranged along the chromosome of *B. cepacia* strain KF1 was drawn. Comparing the relative order of the genes in KF1 with the *Bam*HI and some of the *Sa*II fragments evidenced on the gel used for the restriction analyses of the cosmid, a partial map of pCosGSP (Figure 4.3) was constructed.

The approximate location of the genes could be identified along the length of the cosmid (Figure 4.3.), but only *Bam*HI and *Sa*II sites could be exploited for mapping of the *gsp* gene cluster-containing region of the cosmid. Other restriction sites along the 23.4kb cosmid could not be mapped because the location of the *gsp* genes could not be traced on them solely based on restriction digestion. The cosmid had sufficient genomic

DNA on either side of the gene cluster obtained, it was envisaged that entire *gsp* operon of BG164R was present on the single cosmid isolated from the genomic library.

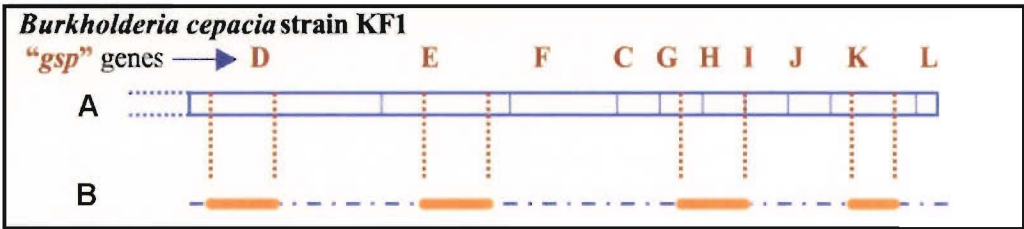


Figure 4.2: [A] *Burkholderia cepacia* strain KF1, which has (84-90%) sequence similarities with BG164R GSP genes. [B] Orange bars represent regions of similarities obtained from sequencing of genomic DNA adjacent to transposon insertion points in clones. (Scale 0.86cm=1Kb)

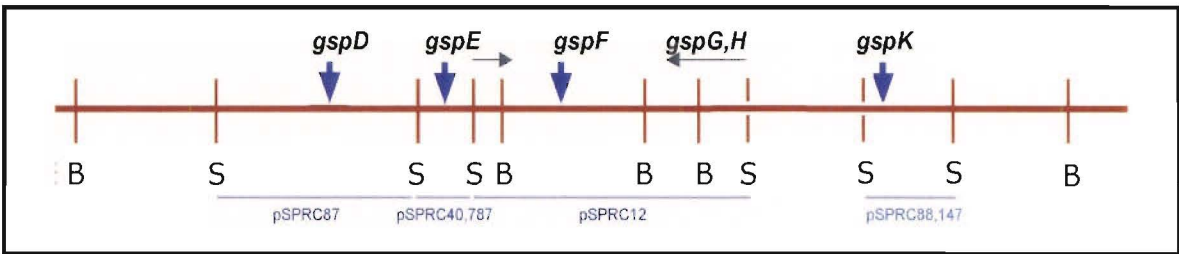


Figure 4.3: Partial physical map of pCosGSP. Blue bold arrows indicate the approximate location of the transposon insertion along the cosmid. B=*Bam*HI, S=*Sal*I. Map not to scale. The clone pSPRC12 was sequenced from both sides. From one end it showed similarities with *gsp*G and H proteins while from the other it showed similarity with *gsp*E. Sub cloning and sequencing of the *gsp*E end led to the identification of the insertion point of the transposon, *gsp*F.

4.3.3. Comparative cosmid stability and mutant rescue experiments.

Complementation analyses described in section 3.3.7, were due to the trans complementation of the mutants in the presence of pCosGSP. To test the stability of the cosmids in the mutant backgrounds in the absence of antibiotic, the comparative stability assay on mushroom slices and in liquid cultures was designed.

Cosmid replication or segregation instability and rate of recombination with the chromosome was calculated after culturing transconjugants in LB broth for 24hr and on mushroom slices without using antibiotics to select for bacteria with the cosmid (Table

4.3 and Figure 4.4.). The putative recombined strains that appeared by allelic recombination by growing in LB broth without antibiotics were tested for the ability to form cavity on mushroom slices (Figure 4.5). Loss of the transposon, mini-Tn5KmlacZ2, from the mutant genomes in the rescued wild type strains were confirmed by Southern hybridisation of the genomic DNA of the rescued mutants with probe constructed from the transposon. Results of the Southern hybridisation of the three rescued mutants are presented in Figure 4.6.

Transconjugant tested.	Cosmid loss (%)		Recombinants (%)	
	In LB media	On Mushrooms	In LB media	On Mushrooms
BG4-12Cos	92.25	34.5	2.75	1.63
BG15-40Cos	96.00	43.75	4.0	2.25
BG12-88Cos	96.75	42.89	3.0	0.85

Table 4.3: Summary of the frequency of tetracycline susceptibility as an indirect measure of cosmid loss and kanamycin susceptibility as an indirect measure of recombination of cosmids in the mutant strains.

The frequency of recombination at the kanamycin insertion sites in the mutants was very low. This result has also obviously been accentuated due to a high rate of cosmid loss in the liquid medium and the actual rate was in all probability slightly higher than that presented. Evidently, the cosmid is more stable in the absence of antibiotics on the mushroom slices than in LB medium. This result could be a reflection of the transconjugants going through a different number of generations in the two backgrounds tested. Although, as presented in chapter II, when the rate of growth of mutants in LB is compared, it is evident that the mutants reach a stationary phase after 10hr growth in LB and steady state in the CFU/mL are observed beyond 10hr. Presence of the transconjugants on the mushroom slices in the assay for longer period could have compensated for this difference in the generation times. However, since the possibility was not addressed in the course of this specific assay it cannot be ruled out.

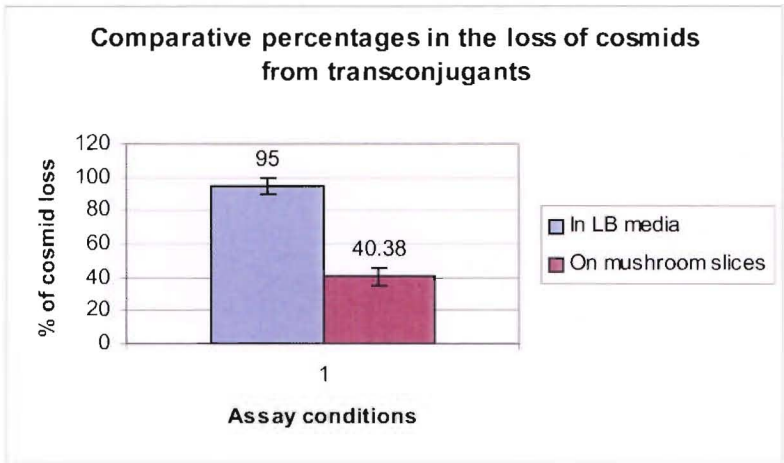


Figure 4.4: Comparative loss of cosmid from the transconjugants in LB and on mushroom slices

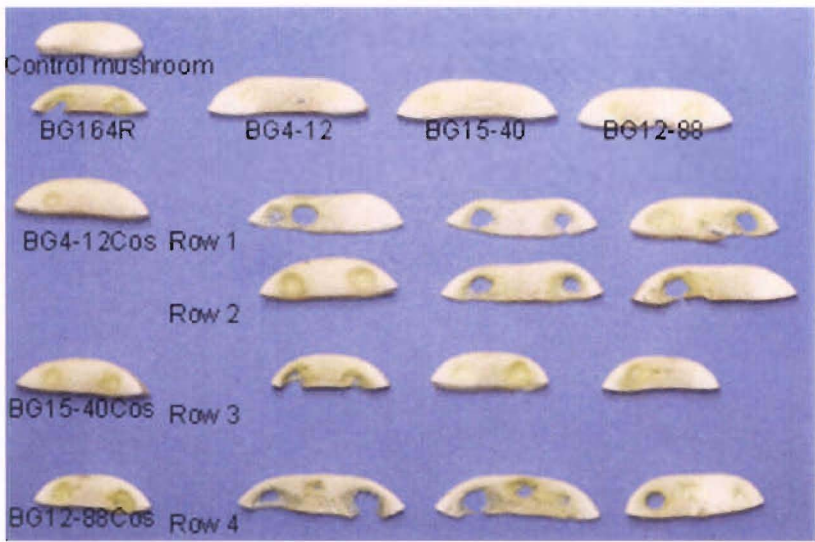


Figure 4.5: Mushroom assay to test the cavity formation by rescued mutants. Row 1:Mutants rescued from BG4-12Cos, Row 2: Mutants rescued from BG15-40Cos, Row 3: Mutants rescued from BG12-88Cos.

In the mushroom assay (Figure 4.5), the transconjugants (BG4-12Cos, BG15-40Cos and BG12-88Cos) show clear cavities in contrast to the mutants (BG4-12, BG15-40 and BG12-88); however, there are clear distinctions between the cavities formed by the transconjugants and those mutants repaired by recombination (in the rows 1,2,3,4 in Figure 4.5). This observation re-emphasises the fact that transconjugants lose a

population of the cosmids, when grown in absence of any antibiotic selection. When the wild type strains are recreated from the mutants, they cause cavities identical to the controls.

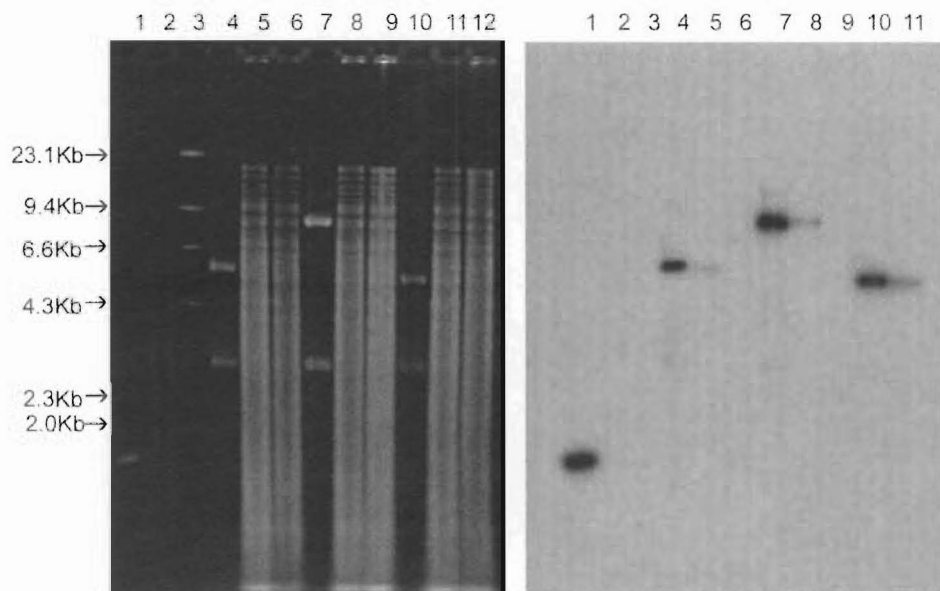


Figure 4.6: [A] DNA gel, [B] Respective film of the southern probed with kanamycin cassette from the transposon. [Lane1] Probe (positive control), [Lane 2] BG164R genomic DNA(control) . [Lane 3] λ /HindIII, [Lane 4] pSPRC40, [Lane 5] BG15-40 genomic DNA. [Lane 6] Rescued mutant from BG15-40. [Lane 7] pSPRC12, [Lane 8] BG4-12 genomic DNA, [Lane 9] Rescued mutant from BG4-12, [Lane 10] pSPRC88, [Lane 11] genomic DNA of BG12-88, [Lane 12] Rescued mutant of BG12-88.

Southern hybridisations (Figure 4.6.) confirmed loss of transposons from the strains, which became kanamycin and tetracycline sensitive in the course of cosmid stability experiments. Since these strains formed cavities of comparable sizes to that of the wild type strains, it can be predicted that events of allelic recombination of the mutants occurred with the cosmid DNA and has resulted in the recreation of wild type strains.

4.3.4. Mapping of pCosGSP by Southern Hybridisation.

Southern hybridisation was used to identify the relative order in which the different fragments of DNA identified from the mutants were arranged along the 23.4kb DNA present in the cosmid pCosGSP. Two probes constructed from different mutant clones were used to confirm the cosmid map (Figure 4.7.). A summary of the DNA bands that hybridised with the probes is represented in Table 4.4.

Enzymes used in the digestion.	Bands in kb, that hybridise with the respective probe.	
	<i>gspF</i> probe	<i>gspK</i> probe
<i>EcoRI</i>	14.5	14.5
<i>BamHI</i>	2.3 and 7.2	6.5
<i>HindIII</i>	16.7	16.7
<i>SalI</i>	4.0	850bp
<i>EcoRI</i> + <i>BamHI</i>	2.3 and 7.2	4.5
<i>EcoRI</i> + <i>HindIII</i>	12.0	12.0
<i>EcoRI</i> + <i>SalI</i>	4.0	850bp
<i>BamHI</i> + <i>SalI</i>	2.3	850bp

Table 4.4: Results of Southern hybridisation of the cosmid DNA with two different probes.

The results confirm the partial map of cosmid pCosGSP (Figure 4.3). The 1.5kb *SalI*-*EcoRI* fragment from the T3 end of the plasmid clone, pSPRC12, served as the *gspF* probe. This fragment has a single internal *BamHI* site. As evident from the autoradiogram, the probe hybridised to two bands in the *BamHI* digested cosmid DNA (Figure 4.7.), a 2.3kb band and the 7.2kb band, which according to the physical map of the cosmid are present contiguously. The *SalI* clone, pSPRC12, has three internal *BamHI* sites in the 4kb genomic DNA on either side of the transposon. The T3 end, which was used to construct the probe, has a single *BamHI* site and the T7 end has two sites. The three *BamHI* sites should theoretically release a 2.3kb and a 700bp band from the 4kb *SalI* fragment, both of which are obvious in the restriction digest and presented in the map. The *gspK* probe, hybridises with a 6.5kb *BamHI* fragment (Figure 4.8.), as predicted from the restriction map data. In the *SalI* digested lanes, the *gspK* probe hybridised with the 850bp (Figure 4.8.) fragment while the *gspF* probe hybridised with the 4kb band (Figure 4.7.).

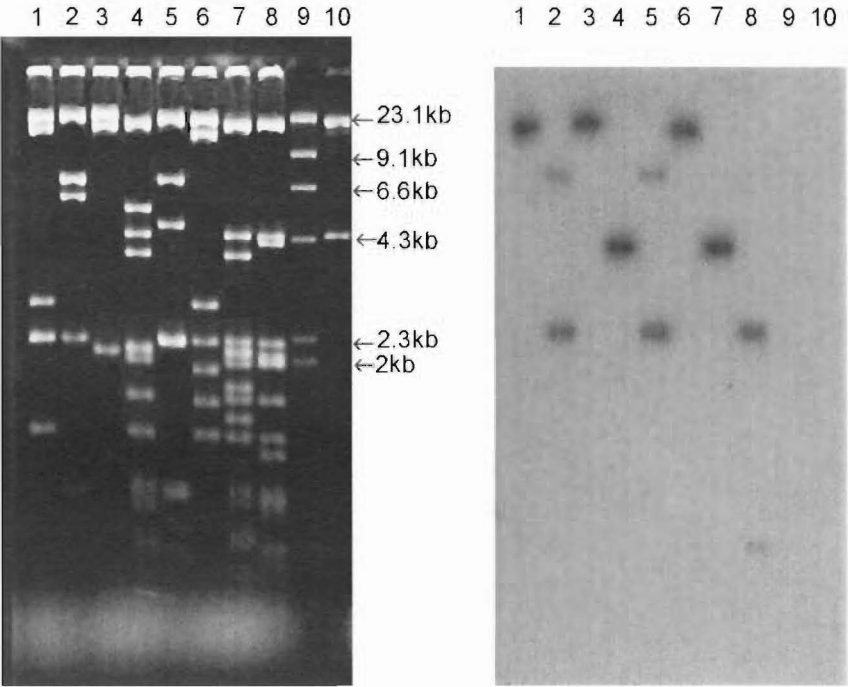


Figure 4.7: pCosGSP digests probed with *gspF* probe. Lanes 1-9) Cosmid digested with different enzymes. [1] *EcoRI*, [2] *BamHI*, [3] *HindIII*, [4] *Sall*, [5] *EcoRI+BamHI*, [6] *EcoRI+HindIII*, [7] *EcoRI+Sall*, [8] *BamHI+Sall*, [9] λ /*HindIII* marker, [10] Negative control, pLAFR₃/*HindIII*+*Sall*

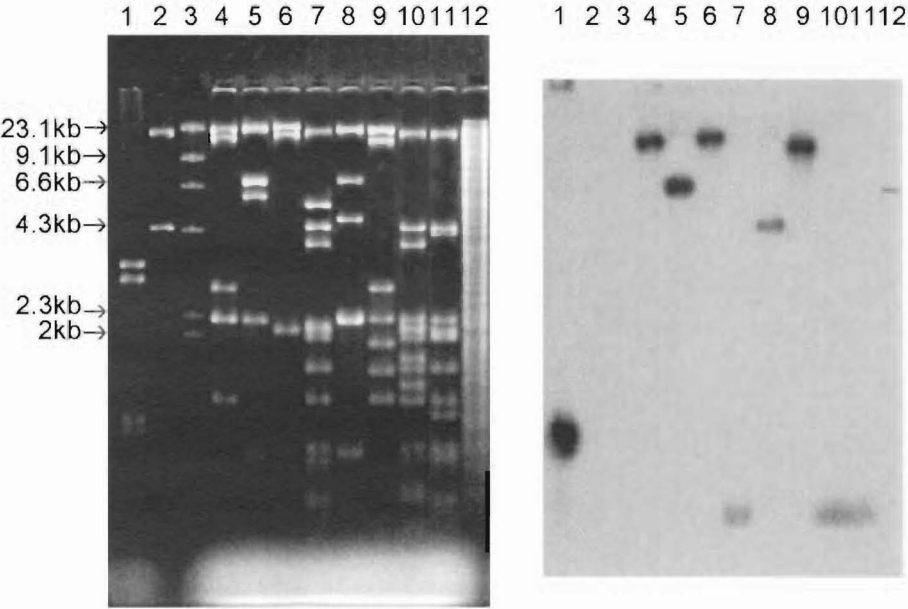


Figure 4.8: pCosGSP digests probed with *gspK* probe [1] Positive control pSPRC12/*HindIII*-*Sall*, [2] Negative control, pLAFR₃/*HindIII*+*Sall*, [3] λ /*HindIII* marker, [4-11] Cosmid digests with enzymes as follows. [4] *EcoRI*, [5] *BamHI*, [6] *HindIII*, [7] *Sall*, [8] *EcoRI+BamHI*, [9] *EcoRI+HindIII*, [10] *EcoRI+Sall*, [11] *BamHI+Sall*, [12] Genomic DNA of BG164R digested with *BamHI*.

The size of the genomic fragment inserted in the cosmid as calculated earlier from *EcoRI-HindIII* digest was 23.4kb. From the results presented, it was evident that both the probes hybridised to similar bands in the *HindIII*, *EcoRI* single digests, and *EcoRI + HindIII* double digests. A second objective of the experiment was to locate a fragment of DNA that would possibly have all the genes of the *gsp* operon and enable us to determine the boundary of the *gsp* gene cluster. Sub-cloning followed by complementation analysis of the 16.7kb *HindIII* and the 12kb *EcoRI-HindIII* fragments was attempted first.

4.3.5. Sub-cloning and analysis of the “*gsp*” gene cluster of BG164R.

Identification of the minimum unit of the cosmid capable of complementing the clones was attempted by sub-cloning of the cosmid. The pBBR1MCS based broad host range *Pseudomonas* cloning vector, pME6001, with gentamycin resistance marker (Antoine & Loch, 1992; Kovach *et al.*, 1994; Maurhofer *et al.*, 1998), was used to sub-clone the genomic DNA inserted in the cosmid pCosGSP in an attempt to isolate smaller fragments of the cosmid capable of complementing the mutants. Shotgun cloning of the *HindIII* fragments yielded both the 16.7kb (pGSP ME H-H 16.7) fragment and the 2kb (pGSP ME H-H 2) fragment. However, in case of *EcoRI-HindIII* cloning, only the 12kb (pGSP ME E-H 12) and the 1.7kb (pGSP ME E-H 1.7) fragments could be cloned out of the six fragments generated by the digestion of the cosmid DNA in 4 independent attempts. Complementation of the mutants with pGSP ME H-H 16.7, were initially tested before any further sub cloning attempts.

Tripartite matings with pRK2013 as the helper plasmid was first used to transfer pGSP ME H-H 16.7 sub-clone into the *gspE*, *gspK* and *gspF* mutants. Transconjugants isolated from matings with all the three different mutants were isolated and tested for complementation of the cavity phenotypes with mushroom bioassays. None of the mutants were complemented with the biggest cosmid sub-clone. This indicates that the minimum complementation unit is larger than the largest clone, although, the Southern hybridisations with probes constructed from two different *gsp* genes showed positive

hybridisation of the same fragment. Considering the fact that the size of the insert DNA in the cosmid pCosGSP is 23.4kb, there was about 6.7kb of DNA missing in the *Hind*III clone. Of the 6.7kb missing DNA, 2kb was sub-cloned as pGSP ME H-H 2, however 4.7kb DNA was attached to the cosmid vector with *Hind*III digestion. These missing portions could simply be a missing promoter on one end of the sub-clone, or could be some other genes in the GSP operon, which were necessary for the efficient functioning of the GSP system. Alternately, there could also be some other gene(s), downstream or upstream the *gsp* operon in *Burkholderia*, which were silenced by polar effects in the mutants and could not be complemented by the sub-clone because of the absence of the corresponding region in the sub-clone. These could include products secreted by GSP or involved in the efficient processing of the products being secreted by GSP machinery and thus were essential for the formation of cavity disease. If some other genes present upstream or downstream the region sub-cloned from the cosmid was necessary for complete complementation, then introduction of another clone containing such regions would result in restoration of wild type phenotypes. Such an attempt was undertaken and will be discussed in section 4.3.7.

4.3.6. End sequence analysis of the cosmid sub-clones.

To analyse possible reasons for the failure of the complementation studies with the pGSP ME H-H 16.7 sub clone and also to orient the sub-clones and the mutant clones in comparison to the published complete *gsp* operon of *Burkholderia pseudomallei* strain 1026b (Fig:4.9) (DeShazer *et al.*, 1999) ends of the cosmid sub-clones were sequenced to identify the genes at the boundaries of each sub-clone.

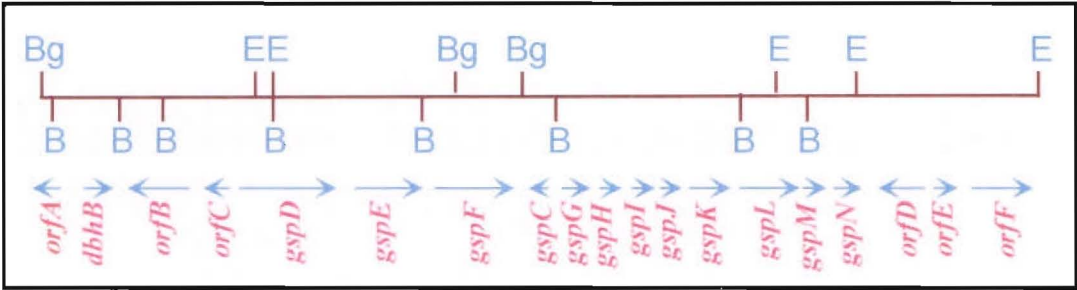


Figure 4.9: *gsp* gene cluster of *Burkholderia pseudomallei* 1026b redrawn from De Shazer *et al.* 1999 (De Shazer *et al.*, 1999). Figure not to scale. B= *Bam*HI, E=*Eco*RI, Bg=*Bgl*II.

Name of the clone	T3 sequence similarity			T7 sequence similarity		
	Gene	% Identity	E-Value	Gene	% Identity	E-Value
pGSP ME H-H 16.7	Last 20bp <i>orfD</i>	100%	0.98	<i>orfB</i>	85%	2e-26
pGSP ME H-H 2	X	NA	NA	<i>dbhB</i>	92%	e-125
pGSP ME H-E 1.7	<i>dbhB</i>	92%	e-107	<i>orfA</i>	84%	4e-12
pGSP KS B-B 2.2	<i>gspC,F</i>	85%,89%	4e-12 7e-55	<i>gspE,F</i>	83%,86%	2e-42, 4e-35
pGSP KS B-B 7.2	<i>orfB</i>	82%	1e-21	<i>gspE</i>	85%	e-143

Table 4.5: Sequence similarity of pCosGSP sub clones with *B. pseudomallei* strain 1026b *gsp* genes.

Interestingly, the two *Hind*III sub clones were identical to genes of *B. pseudomallei* strain 1026b, from one end (Table 4.5). In case of pGSP ME H-H 16.7, a short stretch of 20bp (at the end of T3 sequence read out off the vector) revealed identity to the *orfD* of *B. pseudomallei* 1026b while the rest of the sequence did not show similarity to genes adjacent to the *orfD* genes present in *B. pseudomallei*, namely, *orfF* and *orfE* (Figure 4.9). The two sub-clones based on the end sequences being read off from the vectors were aligned along the physical map of the complete *gsp* operon of *B. pseudomallei*. The sub-clones appear to be two adjacent fragments, as presented in figure 4.7. The 1.7kb *Eco*RI-*Hind*III clone formed a part of the 2kb *Hind*III sub clone, with the *Hind*III end of both the clones showing significant sequence similarity with the *dbhB* gene responsible for coding of a DNA binding protein. The *Eco*RI end sequence of the *Eco*-*Hind* sub-clone was similar to another DNA binding protein, OrfA, present adjacent to the *gsp* operon in case of *B. pseudomallei*.

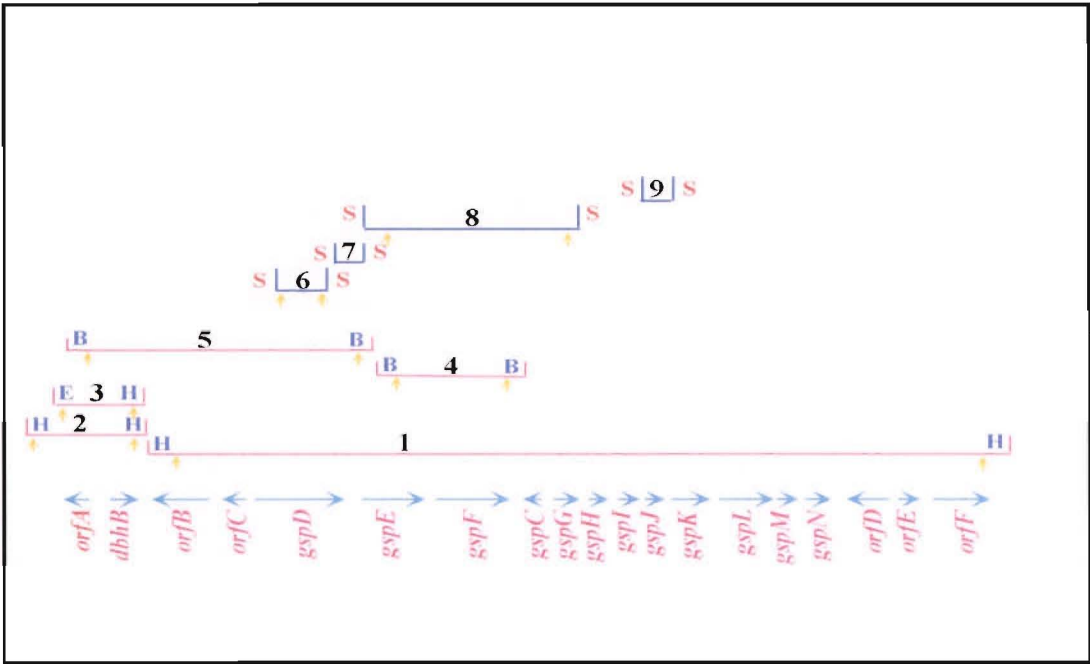


Figure 4.10: Alignments of the cosmid sub clones with the *gsp* operon of *B.pseudomallei* strain 1026b. Blue arrows indicate the alignment of the genes in *B. pseudomallei*, Pink lines indicate the sub clones isolated from the cosmid pCosGSP, while blue lines indicate genomic DNA present in the mutant clones. [1] pGSP ME H-H 16.7, [2] pGSP ME H-H2,[3] pGSP ME H-E 1.7, [4] pGSP KS B-B 2.2, [5] pGSP KS B-B 7.2, [6] pSPRC87, [7] pSPRC40 and 787, [8] pSPRC12 and [9] pSPRC88 and 147. Each orange arrow corresponds to the middle of a 600-800bp sequenced DNA fragment from the end. (Figure not to scale)

Sequence of the two *Bam*HI sub-clones was also found to be very similar to the *gsp* genes of *B. cepacia* KF1 strain. According to predictions, the 2.2kb *Bam*HI sub-clone read into GspC and GspF proteins from the T3 end. Sequence similarities with the published sequences of *B. pseudomallei* is presented in Table 4.4, while it revealed 80% and 88% similarity with *gspC* and *gspF* gene of *B. cepacia*, with an E-value of 2e-07 and 3e-51 respectively. From the T7 end, the sequences showed 82% identity (Expected value=4e-47) with the *gspC* gene and 85% identity (Expected value=9e-07) with the *gspF* genes of *B. cepacia*.

Both pGSP ME H-H 16.7 and pGSP KS B-B 7.2, had similarities to the *orfB* genes of *B. pseudomallei* strain 1026b, the former from the T7 end, while the latter was from the T3 end of the clone. When the sequence of these two genes are aligned they reveal a 99%

similarity (Appendix III), with the *Bam*HI site being at 16bp downstream from the *Hind*III site present adjacent to the *orfB* gene.

4.3.7. Cosmid-plasmid complementation of the mutants

A cosmid-plasmid complementation study was attempted to find out whether the two *Hind*III cosmid sub-clones, when present together in the mutant, had the ability to complement the no-cavity forming phenotype of the mutant. The 2kb *Hind*III fragment from pGSP ME H-H 2 was re-cloned into the compatible plasmid pLAFR₃ to create the cosmid pGSP LA H-H 2. pLAFR₃ belongs to a different replication incompatibility group than pME6001 so both are theoretically stable in the same cell. Triparental matings with the helper plasmid pRK2013 was used to transfer the new cosmid clone pGSP LA H-H 2, into BG4-12ME. Transconjugants were tested on mushroom slices for complementation. None of the transconjugants isolated could complement the mutants in the mushroom slice bioassays.

No complementation of the mutant in the presence of two contiguous fragments containing sub-clones of the cosmid indicated that either the presence of some essential genes downstream the *gsp* gene cluster in the cosmid, or separation of essential DNA sequences (Eg, a promoter site) required for the activation of the mutated *gsp* gene in the *gsp* gene cluster containing sub-clone, pGSP ME H-H16.7. Another possibility is the instability of the pME6001 clone in the mutant background. Such questions need to be addressed in future projects.

4.3.8. Does pCosGSP contain genes of cavity causing principle(s)?

The cosmid pCosGSP was introduced into a *Pseudomonas fluorescence* mushroom isolate (PRC121) to test whether the cosmid, apart from having the secretory genes, also had the structural genes responsible for the production of enzymes/compounds responsible for causing Cavity disease. Pullulanase secretion by *Klebsiella oxytosa* and cellulase and pectinase secretion by *E. chrysanthemi* has previously been reconstituted

in *E. coli*. Cosmid pCosGSP containing a DH5 α clone isolated from the library was tested on mushrooms for the ability to cause any disease like symptoms, but no such reaction was observed on the mushroom tissues. This was more likely due to the fact that *E. coli* cells did not appear to grow on the mushroom slices. Thus, to eliminate the problem of growth of a different strain harbouring the cosmid, pCosGSP was transferred into one of the strains isolated from the button mushrooms, PRC121 (discussed in section 5.4.1.). PRC121 transconjugants with the cosmid did not make cavities on the mushroom slices.

Generally, the *gsp* genes of one species do not complement *gsp* mutants of a second species. Also, there is a specificity of the identification of products secreted by the *gsp* apparatus of each bacterial species. However, there are occasional reports of exoprotein secretion by *gsp* of heterologous hosts, like the secretion of *Aeromonas hydrophila* aerolysin by a marine *Vibrio* strain (Wong *et al.*, 1990) and *P. aeruginosa* elastase by *P. alcaligenes* (de Groot *et al.*, 2001). PRC121 or, *P. fluorescence* was a readily available strain that could be tested in this specific case, as ability of the test strain to grow on mushroom slices would also form a major factor in this situation. Transconjugants isolated by triparental matings were used to set up mushroom bioassays and none of the transconjugants showed any sign of cavity disease.

Inability of the mushrooms to react to any other bacteria harbouring the cosmid pCosGSP could have either been due to the absence of the products responsible for the exhibition of the specific phenotype, alternatively, it could also mean inability of substrate recognition by the GSP in *Pseudomonas fluorescence*. Without knowing what product(s) were supposed to be secreted by the GSP of BG164R, co-relating the ability of products to be secreted by the GSP of other related strains or the reconstitution of the BG164R GSP in heterogenous strains was difficult.

4.3.9. Second mutagenesis screen for isolation of further no-cavity forming mutants.

A second mutagenesis screen was undertaken in an attempt to isolate more no-cavity forming mutants of BG164R, essentially because all mutants isolated up to this point were in the GSP operon.

4.3.9a. Mutagenesis screens and phenotypic characterisation of mutants.

A new group of random insertional mutants were created by mini-Tn5*KmlacZ2* and screened using the standard bioassay system for the loss of the capacity of forming cavities on mushroom pieces. Of the 5000 transconjugants tested, 3 prototrophic mutants were selected for exhibiting the no-cavity phenotype. The *lacZ* fusion status of the transposon in the gene of interest was checked in all the three mutants by plating on an LB plate supplemented with 5-bromo-4-chloro-3-indoyl- β -galactopyranosidase (X-gal). All 3 mutants produced the characteristic blue colour, thereby revealing the formation of active *lacZ* translational fusion in the mutated genes.

BGII-105 had a single flagellum and BGII-106 had two flagella observed by TEM. However, in case of BGII-107 there appeared to be three or more flagella present across several fields of observation, with some rare ones with two flagella. The mutants BGII-105 and BGII-106 had defects in protease secretion while BGII-107 did not have any such defects. The main purpose of this second round of mutagenesis screening was aimed to find out if any other gene apart from the *gsp* gene cluster could be attributed to cavity formation on mushrooms by BG164R using the specific mutagenesis tool and the bioassay system. So, the mutations isolated were cloned into the sequencing vector pBluescript KS⁺ using *Sa*I. Properties of the mutants isolated and the clones created are described in Table 4.6.

Name of the mutant isolated	'lacZ fusion status	Name of the respective clone in DH5 α	Name of the respective plasmid	Total size of insert DNA in the clone	Amount of genomic DNA present
BGII-105	Blue	CMCII-105	pSPRC105	4.4kb	200bp
BGII-106	Blue	CMCII-106	pSPRC106	4.7kb	500bp
BGII-107	Blue	CMCII-107	pSPRC107	7kb	2.8Kb

Table 4.6: Properties of the mutants and the respective clones isolated in the second lot of mutagenesis screening of no-cavity phenotype.

Evidently, two of the clones were quite small, unlike any other clone isolated in the first round of mutagenesis screening experiment and the size of all three clones were quite different to the mutants cloned so far.

4.3.9b. Sequence analyses of the mutant clones and complementation studies.

The sequences were used to search the available database. Two out of three clones had mutations in the GSP genes, one of which was different to the *gsp* genes identified so far. Interestingly the third mutation was in a gene which had significant sequence similarity with the polyketide synthetase (*pks*) gene of *Myxococcus xanthus* involved in the synthesis of the polyketide antibiotic, TA (Simunovic *et al.* , 2003). Details of the similarity search with the sequences are presented in Table 4.7.

Construct number	T3 primer		T7 primer		Possible Gene Mutated
	Length read out	Identity to gene	Length read out	Identity to gene	
pSPRC105	17bp+Tn5	No sim	154+Tn5	<i>E</i>	<i>gspE</i>
pSPRC106	396bp+Tn5	<i>K</i>	97+Tn5	<i>K</i>	<i>gspK</i>
pSPRC107	840bp	<i>pks</i>	800	<i>pks</i>	Polyketide synthetase

Table 4.7: Results of similarity searches with the gene sequences.

Similarity searches revealed that the *gspE* mutation in pSPRC105, was in a 171bp *SalI* fragment, which has not been isolated before. The 17bp genomic DNA sequenced from the T3 end of the vector did not match with any published sequence, either because of the very small amount of information present in it, or because the mutation was at an extreme end of the published *gspE* sequence. Sequence from the T7 end of the clone was 89% similar to *B. cepacia gspE* gene, with an E value of 1e-39, and 87% similar to the *gspE* of *B. pseudomallei* with an E value of 4e-33. The 154bp sequence did not match with any of the partial *gspE* sequences from the clones pSPRC40, pSPRC787, pGSP KS B-B 7.2 and pGSP KS B-B 2.2.

There is 96.63% similarity in the sequences from the T7 end of the clones pSPRC88, pSPRC147 and pSPRC106 and 86.7% similarity from the T3 end. Although sequences up to the transposon insertion point was not achieved in the clone pSPRC147 from the T3 end, based on the data collected from the T7 end, it appears that the clones pSPRC88 and pSPRC106 are similar, indicating that the mutants BG12-88 and BGII-106 are the same.

BGII-107 has a unique mutation in the gene that shows significant sequence similarities both at the DNA and protein levels to the polyketide synthetase gene of *Myxococcus xanthus*. The exact location of the mutant could not be traced in case of the clone as sequencing reactions were done with primers based on T3 and T7 promoters present in the vector. From both the ends, over 800bp sequences were obtained which did not overlap with the end of the transposon. The sequences from the T3 end of the BGII-107 clone showed 83%, 84% and 90% similarity over three stretches of approximately 100bp DNA with E values of 2e-06, 1e-07 and 0.024 to the *Myxococcus xanthus* polyketide synthetase gene (Appendix III). Analyses of the translated amino acid sequence from the 800p DNA sequence in the Blast X server (www.ncbi.nlm.nih.gov/blast) revealed a similarity varying from 45% to 74% with type I polyketide synthase protein of *Bacillus subtilis*. From the T7 end a small stretch of 34bp shows 94% DNA sequence identity with the *Myxococcus xanthus* polyketide synthetase gene.

All the three new mutants were complemented by the cosmid pCosGSP. This indicated the probable presence of the polyketide synthetase gene somewhere along the 23.4Kb genomic insert in the cosmid. Based on results of the Southern hybridisations and sequencing data from the mutant clones, the cosmid sub-clones and the new mutants clones, a restriction map of the cosmid was reconstructed (Fig.4.11), which revealed approximately 5kb genomic DNA downstream the *gsp* gene cluster of BG164R and is possibly the location of the polyketide synthetase gene in the cosmid.

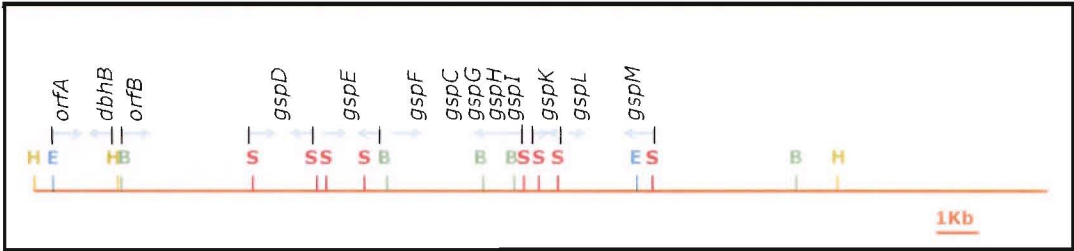


Figure 4.11: Restriction map of pCosGSP genes. The blue arrows indicate the partial sequence of the genes obtained from sequencing of the mutant clones and the cosmid sub-clones. The directions indicate readouts from the T3 and T7 primers, and **not** the direction of transcription of the respective genes. E= *EcoRI*, B=*Bam*HI, H= *Hind*III, S=*Sal*I.

4.4. Discussion.

In this project, screening of 15,000 random mutants yielded 9 prototrophic no-cavity causing mutants. Of these, 8 mutants have the transposon inserted in the different genes of *gsp* operon of BG164R. The only mutant that had a mutation in a different gene was the polyketide synthetase mutant, BGII-107. Complementation of mutants with pCosGSP indicates the presence of the *gsp* as well as the polyketide synthetase genes along the cosmid. While the exact physical location of the *gsp* genes along the cosmid have been established, speculation regarding location of the polyketide synthetase gene needs further genetic verification. It is likely that GSP in *B. gladioli* (BG164R) secretes a product, synthesised by the polyketide synthetase gene of the Cavity disease pathogen.

The 16.7kb *Hind*III sub clone consisted of all the genes of the *gsp* operon, as identified by the Southern bolt analysis, however, it did not complement the mutations. De Shazer *et al.* (1999) from their experiments suggested that the 5' and 3' termini of the *gsp* gene

cluster were the *orfC* and the *orfD* genes in their strain (*B. pseudomallei* 1026b). It does not appear to be the case in BG164R *gsp* genes. End-sequence analysis of the clone suggests that the 16.7kb *HindIII* sub-clone has both the terminal genes present in the case of *B. pseudomallei* 1026b. The result indicates the absence of a promoter or operator gene required for the activation of *gsp* gene cluster in the sub-clone, as a result of which the genes are not transcribed. Alternatively, this could indicate silencing of gene products downstream of the *gsp* operon, in the case of mutants due to polar effects exhibited by mini-Tn5*KmlacZ2*.

Sequence analysis of the cosmid sub-clones and mutant clones used to align the genes reveals the presence of a gap between the *gspM* and the *orfD* genes identified by the end sequencing of pGSP ME H-H16.7. According to the order of the genes in *B. pseudomallei* 1026b, *gspM* is followed by *gspN* gene. The presence of *gspN* gene in the *gsp* operon of BG164R needs to be verified by sequencing of the intermediate region. Notably, *orfD* appears to be the last gene in the cluster in case of the BG164R *gsp* operon, in contrast to that of *orfE* and *orfF* genes present beyond *orfD* in the case of the *B. pseudomallei* 1026b *gsp* operon (Fig 4.9). The boundary of the BG164R *gsp* operon could not be determined by the sub-cloning and complementation analysis.

In the absence of knowledge about the actual products that were responsible for cavity formation, and the fact that the cosmid, pCosGSP, did not exhibit any disease symptoms on mushroom slices when tested from *E. coli* background, restricted the possibility of screening the mutated cosmids for cavity formation in the *E.coli* background. Thus, screening for 'no-cavity' phenotype of the mutated cosmids would be dependent on the ability to create a huge number of mutant *Burkholderia* strains by recombination with randomly mutated cosmids. However, an alternative strategy could be adopted in this case, which would involve saturation mutagenesis of the cosmid, introduction of the mutated cosmid into the existing mutants, and identification of mutant cosmids, that would not complement the cavity formation. This, although was not the best possible alternative, would have given us some indication about the regions of the cosmid necessary for disease formation. A problem associated with this saturation mutagenesis approach was the fact that BG164R is resistant to a wide range of common antibiotics. A mini-Tn10 transposon derivative with a gentamycin resistance gene cassette in a

suicide vector (pBSL177), pBSL182 (Alexeyev *et al.* 1995; Alexeyev & Shokolenko, 1995) was used in mutagenesis experiments, but transposition could not effectively induced. The alternative strategy of sub cloning of fragments from the cosmid, to isolate smaller complementing fragment appeared to be a quicker approach to delimit the boundaries of *gsp* gene on the cosmid, as southern hybridisations indicated a high probability of the presence of the *gsp* gene in the 16.7kb *Hind*III fragment.

The polyketide synthetase gene of *Myxococcus xanthus*, codes for a unique polypeptide with combined enzymatic activities of non-ribosomal peptide synthetase and type I polyketide synthase (Paitin *et al.*, 1998). Thus, when analysed at the translated query levels, significant similarity was observed with the *Bacillus subtilis* type I polyketide synthase. The *Myxococcus xanthus* polypeptide assembles amino acids by peptide bond formation in energy dependent manner by virtue of the non-ribosomal peptide synthetase enzyme activity, which covalently adds acetate or propionate units as by the type I polyketide synthase function (Simunovic *et al.*, 2003). The final outcome of the complex enzyme in *Myxococcus xanthus* is the synthesis of a macrocyclic polyketide antibiotic, TA, which has an unsaturated $\alpha\beta$ -methaoxy group and is formed from methionine, glycine and acetate precursor molecules. The antibiotic is known to be an inhibitor of cell wall synthesis by interfering with the lipid-disaccharide-pentapeptides aggregation (Paitin *et al.*, 1998).

Polyketides form a versatile group of natural products, essentially secondary metabolites, produced by bacteria, fungi and plants. The activity range of the different compounds varies from being anticancer agents to antibiotics, from immunosuppressants to mycotoxins. The question that arose was whether the mutant had the capacity to inhibit fungal hyphae, a well-exhibited character of *B. gladioli* pv. *agaricola*, BG164R. Fungal assays revealed no change in the characteristic of the mutant (Figure 4.12), although the mutant has lost the capacity of forming cavity on mushroom tissues.

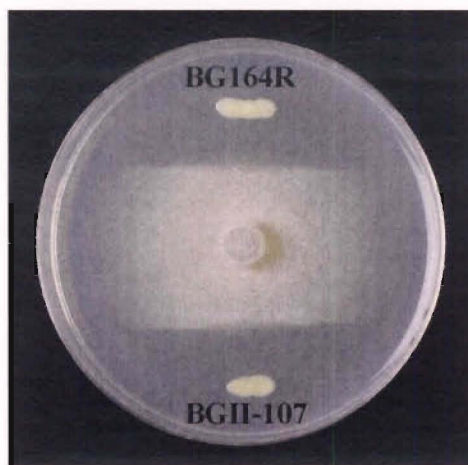


Figure 4.12: Inhibition of *Gaeumanomyces graminis* mycelia by the polyketide synthetase mutant, BGII-107

The isolation of 8 GSP mutants in the course of mutagenesis certainly indicates the involvement of extracellular enzymes in the formation of cavity disease in mushrooms. GSP has been associated with the secretion of toxins in many species, including toxin A secretion in *Pseudomonas* (Mc Vay & Hamood, 1995), aerolysin in *Aeromonas* (Wong *et al.*, 1990; Howard & Meiklejohn, 1995) and the cholera toxin in *Vibrio* (Sandkvist *et al.*, 1997). However, in all these cases, the toxins are basically proteins in nature, and specifically recognised by the respective *gsp* secretin. The general secretory pathway, is a system dedicated to the secretion of proteins and hence, the possibility of a gene product being synthesised by an enzyme that has mixed function of non-ribosomal peptide synthetase and type I polyketide synthase cannot be ignored.

Since the polyketide synthetase mutant is complemented by the cosmid, it is predicted that the genes are situated downstream from the *gsp* gene cluster in this species. The prediction needs further verification; however, the questions that remain unanswered are what is the chemical nature of the product being produced by the polyketide synthase gene linked to cavity disease of mushrooms? On consideration of the possible roles played by the polyketide synthetase genes in formation of cavity disease, two probable mechanisms can be proposed: (i) as a signalling molecule involved in quorum sensing (ii) in the production of surface bound components of the bacterial cells, which play a role in the bacterial pathogenesis. It has been demonstrated by DeShazer *et al.* (2001) that the *wcbR* gene with putative type I polyketide synthase functions plays a role in the

biosynthesis, export and translocation of polysaccharides necessary for the formation of capsules (DeShazer *et al.*, 2001). Mutation in the *wcbR* gene, which has polyketide synthase function, reduces *Burkholderia mallei* pathogenicity in the formation of glanders disease in Syrian hamster and mice models. Could such a phenomenon also be working in the formation of cavity disease on mushrooms?

4.5. Concluding Remarks.

An efficient random mutagenesis system and a genomic library to study genes of BG164R have been established in the course of this study. Isolation and identification of more mutations would certainly lead to the identification of the products involved in the expression of Cavity disease. Alternatively, secretory mutants isolated in the course of this study could be exploited in a reverse genetics approach to identify the secreted gene products involved in the expression of disease symptoms.

Chapter V

Initiation of Cavity disease on mushrooms.

5.1. Introduction.

The term ‘disease’ is derived from old French, which literally means ‘a departure from normal or easy living’ (Broden & Guthmiller, 2003). Over the years, the definition has undergone many changes and current usage implies the ‘manifestation of damage to host’ that results from host-microbe interactions. Expression of disease symptoms in a host is the final outcome of a series of sequential steps, starting from the initial establishment of the pathogen, followed by the colonisation of the host tissue and ultimately leading to the expression of disease.

In order to successfully establish themselves as pathogens, microorganisms not only overcome the defence mechanisms of the host but also compete with other microorganisms in the immediate environment, the rhizosphere or phyllosphere of the host tissue. Mushroom sporocarps harbour a population of non-pathogenic commensals (Moquet *et al.*, 1996). Expression of some disease processes in mushrooms has been reported to involve secondary infection in the presence of a primary pathogen (Inglis *et al.*, 1996a; Moquet *et al.*, 1996). In the commercial cultivation of mushrooms, the fruit bodies grow from mycelia that are seeded on compost beds that have been reported to consist of microbial populations in them (Godfrey, 2003). The induction of sporocarp formation from mycelia needs a signal from *Pseudomonas putida* to remove the “self inhibitory compounds” produced by the growing mushroom mycelia into its environment below the threshold level (Rainey, 1989). *P. putida* also forms a dominant population of the compost bed used in the commercial cultivation of mushrooms (Rainey, 1989). Sporocarps of mushroom thus provide microenvironments to the pathogen, analogous to the phyllosphere and rhizosphere organisms in case of plants. Studies on the effect of bacteria present in the microenvironment of mushroom

sporocarps in Cavity disease initiation by BG164R forms one of the main bases of this chapter.

Besides being the Cavity disease pathogen, the strain BG164R, has been associated with the inhibition of filamentous phytopathogens as *Fusarium oxysporum* and *Trichophyton sp.* (Young, 1992). The chapter also describes experiments designed to further explore the host range of the pathogen, in an effort to biologically characterise the strain.

5.2. Material and Methods.

5.2.1. Bacterial strains used.

Strains and plasmids	Genotype or description	References
<i>Burkholderia gladioli</i> pv. <i>agaricola</i> .		
BG164R	wildtype, cav+, Af+, Rif ^R , Prot+	This study
<i>Pseudomonas aureofaciens</i>		
PA147-2	wildtype, Af+, Bfm+, Rif ^R , Cm ^R , (10µg/mL)	Carruthers <i>et al.</i> 1994
<i>Ewingella americana</i>		
PRC120	non-pathogenic, wild type mushroom isolate, Prot+	This study
<i>Pseudomonas fluorescens</i>		
PRC121	non-pathogenic, wild type mushroom isolate, Prot-	This study
PRC122	non-pathogenic, wild type mushroom isolate, Prot-	This study

Table 5.1: List of strains used in the experiments described in Chapter 5.

5.2.2. Media, reagents and antibiotics.

All media and reagents used in this study were prepared as described in Appendices I and II. Concentrations of the antibiotics used to maintain the selective pressure on the strains are as mentioned in Appendix I, unless otherwise stated.

5.2.3. Bacteriological methods and antifungal assays.

The bacteriological methods and cultural conditions used for BG164R and PA147-2 were as stated in section 2.2.3. The strains of *Ewingella americana* and *Pseudomonas fluorescens*, isolated from mushroom sporocarps were grown under aerobic conditions, in a 30°C incubator. Individual single colonies of the *E. americana* and *P. fluorescens* strains appeared within 20hr of incubation. The antifungal assays were set up according to the method stated in section 2.2.7h.

Initially, antifungal assays were set up in different media to identify a media best suited to exhibit the antifungal characteristics of BG164R. Potato dextrose agar (PDA), Tris Buffered potato dextrose agar (TB-PDA) maintained at pH 7.6 and corn meal agar (CMA) were tested for the purpose. The growth of BG164R was monitored over a period of four days in each media and compared to another antifungal pseudomonad, *P. aureofaciens*, strain PA147-2.

5.2.4. Experiments designed to determine the inoculum size required to initiate Cavity disease.

To test the influence of inocula on the initiation of Cavity disease, stationary phase cultures grown overnight for 18hr and arising from well-isolated single bacterial colonies were serially diluted to a factor of 10^8 , and 5µL drops from all dilutions were spotted in triplicate onto mushroom slices and onto LB plates to enumerate the inoculum size. Inoculated slices were incubated up to 120h and disease progression was monitored at regular intervals. Data collected from four repeats of the assay were used

to determine the minimum number of cells necessary for disease initiation within 16hr under standardised mushroom assay conditions described in section 2.2.5.

5.2.5. Assay to monitor the growth of BG164R on mushroom tissues.

To select the best process for the extraction of live bacterial cells from mushroom tissues, methods like sonication and vortexing of samples, with varying intensity and time to retrieve fixed number of cells were tried. Based on these preliminary experiments it was established that vortexing of the mushroom pieces with 100 μ L of 1 x M63 minimal salt solution was the most efficient method for the extraction of live cells.

To measure the growth of BG164R over time on mushroom tissue, sporocarp tissues were cut into 1cm x 1cm x 2 mm slices and inoculated with drops of suspension cultures containing estimated number of bacteria. At regular intervals of time, bacteria were extracted from the mushroom slices by continuous vortexing the tissue samples in 100 μ L of minimal salts (M63), in an Eppendorf tube for 1min. The suspensions were serially diluted and plated on LB plates supplemented with Rifampicin to enumerate BG164R and select against the growth of the other (*rif^S*) microflora present in the mushroom pieces.

5.2.6. Mushroom bioassays to test the effect of mixed populations on the capacity of disease formation by BG164R.

Eighteen hour old saturated cultures of BG164R, PRC120, PRC121, PRC122, and a control strain, *Pseudomonas aureofaciens* PA147-2 (that does not show any disease on mushrooms), were diluted to an optical density of 0.5 at 600nm wavelength. Hundred μ L aliquots of diluted samples from the three mushroom isolates were separately mixed with BG164R and PA147-2. Mixed samples were centrifuged, resuspended in a final volume of 100 μ L of 1 x MS and used as the stock inoculum. Five microliter aliquots were spotted on to mushroom slices in duplicate, incubated at 30°C and disease progression recorded at regular intervals over a 3-day period. The ratio of BG164R and

mushroom isolates used as inoculum was estimated from viable plate counts on LB plates and LB plates supplemented with rifampicin, confirming roughly 1:1 ratio in the case of all the mixed samples and an inoculum size of approximately 10^7 cells per bacterial type in the mixtures.

5.2.7. Mushroom bioassays to determine the inhibition of BG164R by PRC120.

Mushroom slices were inoculated with a mixed population consisting of BG164R and PRC120, bacteria retrieved from the slices by vortexing at 0hr and 6hr were enumerated. In a control set-up, mushroom slices were inoculated with BG164R only and the concentration of cells estimated after both 0hr and 6hr. The total number of cells present in the mixed population was counted by plating the mixture on LB plates, while the number of BG164R cells in the mixed population was selected by plating the mixture on rifampicin plates.

5.2.8. Extraction of endogenous bacteria from mushroom tissues.

Caps of button mushrooms were washed twice with sterile distilled water and internal pileus tissue were aseptically collected under sterile conditions in a laminar airflow chamber. From four different sporocarps, 0.3g of internal tissue samples were macerated with a sterile glass rod in 100 μ L of minimal salts (MS) and the macerate spread on an LB plate and incubated at 30°C for 20hr to facilitate growth of bacterial colonies. Preliminary identification, based on colony morphology, revealed the presence of three distinct colony types, which were enumerated and re-purified by streaking on fresh LB plates. These three strains were thereafter distinguished by Gram's stain, oxidase test and designated as PRC120, PRC121 and PRC122.

5.2.9. *In vitro* plate assays to test whether the antifungal compound(s) are produced by BG164R on induction by growing fungal mycelia.

Plugs of agar were taken from (i) the zone of inhibition of fungal mycelia on a PDA plate previously used for the antifungal assay, (ii) around the zone of growth of BG164R colonies on a PDA plate, which did not have any fungus growing on it (iii) around the fungal mycelia growing on a PDA plate without any bacteria growing on it, and served as the test blocks. In the actual experiment, agar plugs were taken out from around a young culture of *Gaeumanomyces graminis* growing on a PDA plate were replaced with the three plugs of agar listed above. The fungal plate with plugs of replaced agar was incubated at 25°C and the effect on the growth of the fungal mycelia observed over two days.

5.2.10. *In vitro* plate tests to determine the capacity of inhibition of a lawn of test bacteria by an antagonist.

Fifty µL aliquots of 18hr old cultures of test strain were added to liquid water agar (Appendix I) held at 40°C and overlayed onto pre-warmed MA plates. The overlay was allowed to set at RT under sterile conditions and fixed volumes of the putative inhibitor strain(s) were spotted onto the overlay plates. Test plates were incubated at 30°C incubator and the zone of clearing of the bacterial lawn was accepted as a measure of the capacity of inhibition.

5.3. Results.

5.3.1. Size of inoculum plays a significant role in the initiation of Cavity disease.

The effect of inocula on the initiation of Cavity disease was tested to determine whether Cavity disease initiation was a cell density-dependent process. A minimum concentration of approximately 2.4×10^5 cells of BG164R was required for disease symptoms to be observed within 32hr. Increasing the inoculum size advanced onset of

the disease. However, if the inoculum size was reduced to 20-24 cells, no disease symptoms were observed even after 120hr of incubation (Table 5.2.).

Dilution factor	Estimated Inoculum size	Symptom observation (Hours after inoculation)							
		16	32	48	64	80	96	112	120
10 ⁰	24X10 ⁶	*	**	***+	x	x	x	x	x
10 ²	24X10 ⁴	-	*	**	***+	x	x	x	x
10 ⁴	24X10 ²	-	-	-	-	H	H	H	A
10 ⁶	24	-	-	-	-	-	-	-	A
10 ⁸	-	-	-	-	-	-	-	-	A

Table 5.2: Cavity formation as a function of inoculum size. Legends: [*] cavity formation, [**] massive tissue degradation, [***+] Complete tissue degradation of mushrooms, [-] No reaction observed, [H] Hypersensitive reaction in 30% of the mushroom slices, [A] mushroom slice starts degenerating assay terminated, [X] tissue disappearance due to excess degradation by the pathogen.

Often only a few potentially pathogenic bacteria can colonise a host and multiply to reach the threshold concentration required to cause a disease (Atkey *et al.*, 1992). In the case of BG164R, 20-24 cells were unable to cause observable symptoms of disease even after 120hr incubation. A possible hypersensitive reaction was observed after 80hr incubation on the slices inoculated with 24X10². These reactions were distinctly evident on the surface of the mushroom tissues but did not have any similarities to BG164R infection of mushroom. The observation was unchanged even after extended periods of incubation (till 120hr). The tissue samples could not be incubated beyond 120hr because the test slices started degenerating. Generally, tissue degradation was such that it would obscure disease symptoms after 5days so whether inocula of roughly 2,400 cells could cause disease on further incubations could not be concluded. Interestingly, the tissue slices inoculated with approximately 24 cells did not show any signs of even hypersensitive reactions after 120hr of incubation.

5.3.2. Effect of the size of inocula on the growth rate of BG164R on mushroom tissues.

The growth rate of BG164R on mushroom tissue was monitored over time. Sporocarp slices were inoculated with approximately 9x10² and 4x10⁴ CFU/mL. The BG164R titre was enumerated at regular intervals by extracting the bacteria from the tissues.

The population of bacteria increased over a period of 36hr (Figure 5.1.). No differences were observed in the *in vivo* growth rate on tissue slices compared to *in vitro* growth in liquid broth. By 30hr, all populations incubating on mushroom tissues converged onto the same concentration. No disease symptoms developed in the first 12hr of incubation on mushroom tissues from either starting concentrations of the pathogen. Signs of initial tissue degradation appeared after 36hr starting from the inoculum of 4×10^4 CFU/mL, and the cavity was observed in both inocula when the bacterial population reached a cell density of 4×10^9 CFU/mL, thereby re-establishing a density-dependent disease initiation by the pathogen.

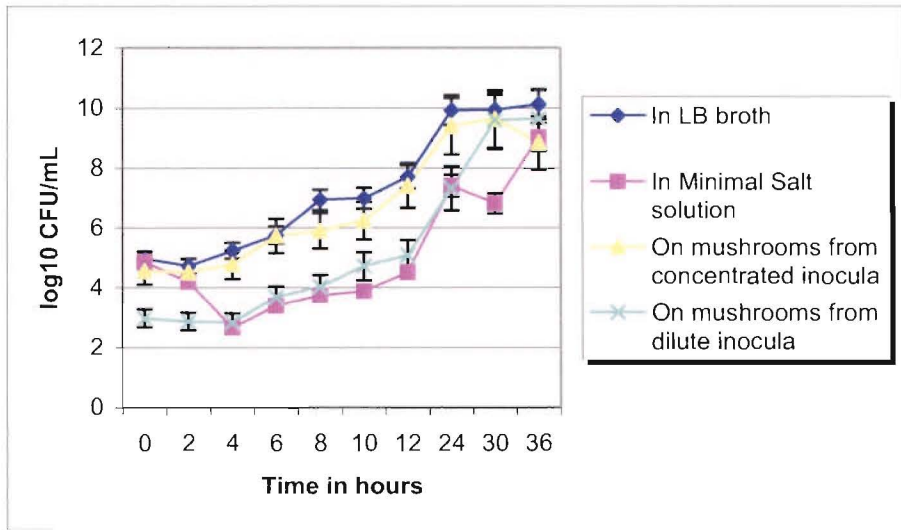


Figure 5.1: Differential growth of BG164R on different substrates and on mushroom tissues from different starting inocula.

There were two problems associated with this assay system. A significant loss of 10-20% bacteria inoculated was realised during extraction from tissue samples. To compensate for the loss, the minimum initial inocula had to be quite high (9×10^2). An inoculum of 20 cells was run in parallel, but the titre decreased over time. Significant observations from the above experiment were - (i) Initial loss of bacteria in the interim period of inoculation and extraction from samples in case of 0hr readings. (ii) When 20 cells were used as inocula there was surprisingly inconsistent number of cells being retrieved from the mushroom tissues with increasing time. Inhibition of the BG164R by

non-pathogenic bacteria, which forms the natural microflora in mushroom tissues, was hypothesised to be the reason for such observations.

5.3.3. Isolation of endogenous bacteria from mushroom tissue.

Bacteria in button mushrooms were isolated and categorised prior to investigating the involvement of such endogenous mushroom bacteria in the initiation of Cavity disease. Three distinct colony types were observed in a preliminary identification of the bacteria present in the mushrooms (being supplied by a fixed farm in Christchurch). Quite naturally, the concentration of the three types of bacteria varied when extracted from a fixed weight of tissue samples arising from different sporocarps (Fig: 5.2). They were designated as PRC120, PRC121 and PRC122. Distinguishing features of these three isolates are presented in Table 5.3.

Character	PRC120	PRC121	PRC122
Colony Morphology	Brown with a small papilla	Small, round, white and transparent	White, round, opaque
Gram's Stain	Negative	Negative	Negative
Fluorescence	Negative	Negative	Positive
Oxidase Test (OX reagent in API20NE)	Negative	Positive	Positive
Antibiotic Sensitivity	Amp ¹⁰⁰ , Nal ¹⁵ , Kan ⁵⁰ , Strep ⁵⁰ , Tet ¹⁵ Gent ³⁰ , Chl ³⁰ Rif ⁵⁰	Kan ⁵⁰ , Strep ⁵⁰ , Tet ¹⁵ Gent ³⁰ , Chl ³⁰ Rif ⁵⁰	Kan ⁵⁰ , Strep ⁵⁰ , Tet ¹⁵ Gent ³⁰ , Chl ³⁰ Rif ⁵⁰
Antibiotic Resistance		Amp ¹⁰⁰ , Nal ¹⁵	Amp ¹⁰⁰ , Nal ¹⁵

Table 5.3: Distinguishing features of three mushroom isolates

The possible interactions of these bacteria with BG164R in the initiation of Cavity disease, was investigated in a number of ways. Firstly, sterilisation of mushroom sporocarps was attempted, with the intention of observing effects of BG164R monoculture on it. Sporocarps were exposed to UV and Gamma irradiations but neither treatment proved to be effective. Sterilisation of mushroom tissues by soaking in rifampicin solution was also attempted and a marked reduction in the ability of BG164R

to cause disease on mushroom pieces was noticed. But, due to problems associated with the re-appearance of endogenous strains of bacteria from mushroom over time from the rifampicin treated mushroom samples, the assay could not be entirely relied upon. Thereafter, experiments were designed using mixed populations of the indigenous bacteria and BG164R.

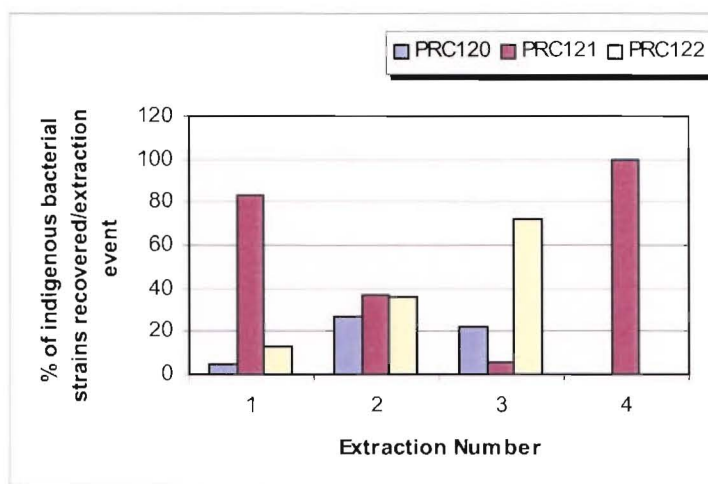


Fig 5.2: Relative abundance of endogenous bacteria present in mushroom sporocarps.

5.3.4. Endogenous mushroom bacteria affect the initiation of Cavity disease.

No disease symptoms appeared on mushroom slices challenged with mixed inocula containing BG164R and PRC120 in 1:1 ratio (approx 10^7 cells for each type). Mixed inocula containing one or more of the other bacterial isolates together with PRC120 and BG164R showed a marked reduction in disease intensity. Even after 3 days of incubation there was no sign of disease symptoms caused by BG164R in the presence of PRC120. This indicated that PRC120 inhibited the virulence of BG164R, which normally caused symptoms at a titre of 2.4×10^7 cells within 16hr (Table 5.2). The indication was confirmed by re-isolation of the total population of bacteria from mushroom tissues inoculated with mixed bacterial populations of PRC120 and BG164R. A marked reduction of the BG164R population after 6hr of co-incubation with PRC120 was observed on the mushroom slices (Figure. 5.3.), while the BG164R monoculture increased over time. However, since we have not been able to challenge sterile mushroom pieces with the mixed inocula, it is difficult to rule out the possibility

of other mushroom isolates, namely PRC121 and PRC122 also having a synergistic effect in the expression of Cavity disease.

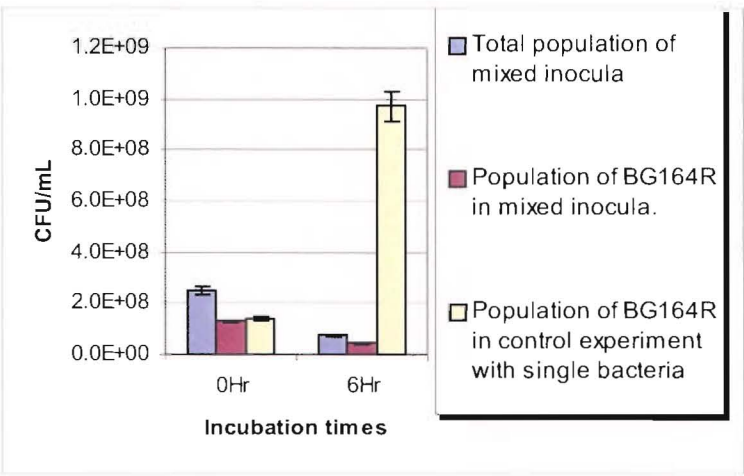


Figure 5.3: Effect on population of BG164R on co-inoculation of with PRC120 on mushroom slices.

5.3.5. Interaction between PRC120 and BG164R.

The inhibition of BG164R by PRC120 was further confirmed using an *in vitro* assay. In an overlay test (section 5.2.10), a lawn of BG164R on minimal agar plate was inhibited by PRC120 (Figure 5.4), thereby confirming the *in vivo* observation on mushroom slices reported in section 5.3.4.

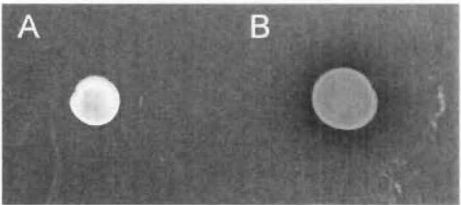


Figure 5.4: Inhibition of BG164R lawn by PRC120. [A] Control colony, which does not inhibit the lawn, [B] PRC120 creating a zone of inhibition on the lawn of BG164R.

5.3.6. Identification of the antagonist and other endogenous bacteria extracted from sporocarps of white button mushrooms.

PRC120, was identified as *Ewingella americana*, by its 16s rDNA sequencing. The other two isolates, namely PRC121 and PRC122, were identified as strains of *Pseudomonas fluorescens*. These results were further confirmed by Biolog tests done at the National Plant Pest Reference Laboratory, Ministry of Agricultural and Forestry, New Zealand. PRC120 was the first report of a New Zealand isolate of *E. americana* from mushrooms, and has been deposited in the New Zealand Culture Collection (accession number of NZRM 4225). *E. americana* has been reported to be the causative agent of internal stipe necrosis in mushroom (Inglis *et al.*, 1996a). Interestingly, PRC120 isolated from button mushrooms in New Zealand does not cause any necrotic symptoms on mushroom stipes or sporocarps and forms a natural population of microbial flora of the mushroom sporocarps.

5.3.7. Interaction of BG164R with other phytopathogenic filamentous fungi.

To identify whether the antifungal activity of the Cavity disease pathogen was restricted only to *Fusarium oxysporum* and *Trichophyton sp.* reported by Young (1992), a number of filamentous phytopathogens were tested by the antifungal assays on PDA plates because they best supported the antifungal activities of BG164R. BG164R inhibited *Fusarium oxysporum* (wilt disease in plants), *Fusarium solani* (sudden death disease in soy bean), *Gaeumanomyces graminis* (Take-all disease in wheat, oat and barley), *Phytophthora megasperma* (root and stem rot of Alfalfa) and, *Rhizoctonia solani* (damping off and sheath blight of tobacco plants) (Figure 5.4.). The best and most drastic inhibition was observed in the case of *Gaeumanomyces graminis* and has been used in all the assays to test the capacity of inhibition of filamentous fungi in all the genetic experiments described earlier.

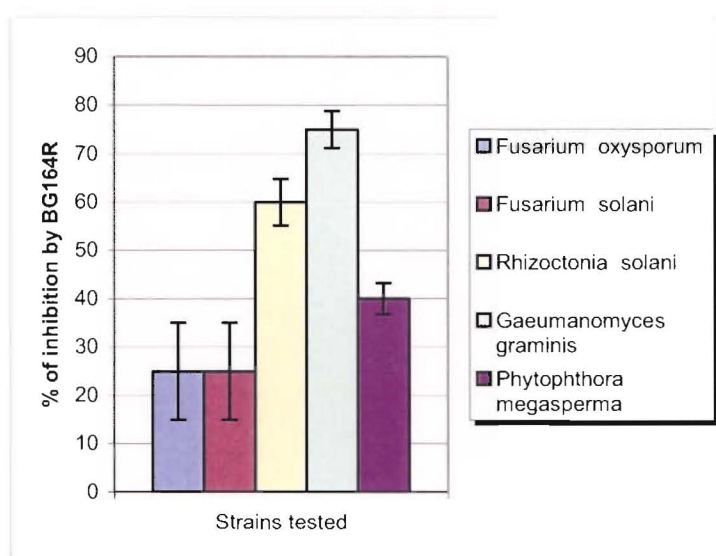


Figure 5.5: Comparative suppression of Phytopathogenic fungi by BG164R.

Mushroom mycelia were thereafter challenged with BG164R. However the assay had to be done on Compost Malt Agar (Appendix I), as it best supports the growth of mushroom mycelia. As expected, BG164R clearly inhibited *Agaricus* mycelia too.

5.3.8. The antifungal compound(s) is constitutively produced by BG164R.

To test whether the production of antifungal compound(s) by BG164R was induced by the growing fungal mycelia (section 5.2.9), *Gaeumanomyces graminis* was challenged with antifungal compounds in the absence of live BG164R cells (Figure 5.6.). Plugs of agar from around BG164R, irrespective of whether it had the fungus growing around it, were able to inhibit the growth of *Gaeumanomyces graminis*.

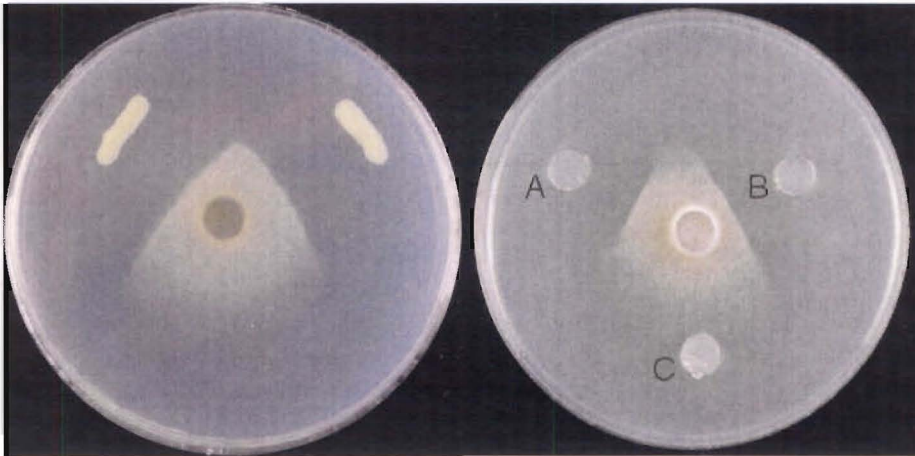


Figure 5.6: **Left:** Control plate showing inhibition of 3 day old *Gaeumanomyces graminis* by BG164R. **Right:** Inhibition of BG164R with plugs of agar taken from three different plates. [A] Plug from plate with only BG164R growing on it. [B] Plug taken from zone of inhibition of fungus from the 3 day old assay plate. [C] Plug of agar taken from plate with only fungi growing on it.

5.3.9. Does BG164R inhibit common yeasts?

Capacity of BG164R to inhibit common yeasts such as *Candida albicans*, known for its ability to cause Candidiasis, and *Saccharomyces cerevisiae*, commonly known as Baker's yeast was tested by overlay tests (section 5.2.9) on PDA plates.



Figure 5.7: Inhibition of *Saccharomyces cerevisiae* lawn by BG164R, and its products. [A] control PDA plugs, [B] colonies of BG164R, [C] Plugs of PDA containing secretory products of BG164R.

S. cerevisiae inhibition was assayed (Figure 5.6.) by live bacteria as well as plugs of agar containing secretory products of BG164R. In contrast, *C. albicans* failed to inhibit BG164R lawn.

5.4. Discussion.

The density-dependent induction of disease by BG164R is a novel observation. In addition, the list of fungal phytopathogens that could be biologically controlled by BG164R, has been extended beyond that reported by Young *et al.* in 1992. It has also been proved that the antifungal compound(s) involved in the inhibition of *Gaeumanomyces graminis* mycelia are constitutively produced by BG164R. The strain is also active against *S. cerevisiae*.

It was initially predicted that if the inhibition of fungal mycelia was an effect of localised degradation of the growing mycelia tip by BG164R, Cavity disease formation in mushrooms could also be affected by the same group of compounds. It was demonstrated, in section 2.3.14, that Cavity minus mutants can still inhibit fungal mycelia, especially the mushroom mycelia. It has also been demonstrated that antifungal-reduced mutants still cause the cavity on mushroom slices. This suggests that Cavity formation and inhibition of filamentous fungi are controlled by different groups of compounds.

Isolation of a natural antagonist of BG164R from mushroom sporocarps supports the concept of the presence of 'Polymicrobial interactions' in the cap tissues in mushroom sporocarps. Polymicrobial interactions (Brogden & Guthmiller, 2003), as opposed to the conventional idea of a single pathogen causing a disease in a host, is an evolving concept (Casadevall & Pirofski, 1999, 2000). Mushroom diseases studied so far have only been attributed to single pathogens (discussed in the introductory chapter), although the presence of a range of non pathogenic bacteria in mushroom sporocarps is well known. Secondary infections by *P. fluorescens* have been noted in the case of internal stipe necrosis (ISN) caused by *E. americana* (Inglis *et al.*, 1996a) and brown

blotch disease of mushrooms caused by *P. tolaasii* (Moquet *et al.*, 1996), but this has not been claimed as the outcome of polymicrobial interactions. Although a similar interaction among pathogenic and non pathogenic forms of *P. syringae* on the phyllosphere in the formation of bacterial blight in soybean (May *et al.*, 1997) has been reported, in general, there is a paucity of reports on the effects of polybacterial interactions in the formation of plant disease. Frequently reported examples of interactions in the formation of plant diseases are polyviral synergisms (Vance, 1991; Anjos *et al.*, 1992; McKern *et al.*, 1992; Pruss *et al.*, 1997).

Diseases in plants due to mixed viral infections are very common. Individual components of such infections act either synergistically or antagonistically in the expression of disease symptoms. A common example is the increased concentration of non-poty viral genomes and symptom expression in the case of mixed viral infections with poty viruses, as demonstrated by Pruss *et al.* (1997) and Anjos *et al.* (1992). Bacterial diseases of plants, in contrast, generally support the classical concept of disease formation by a single pathogen. It is likely, however, that some plant diseases are the results of interactions between a wide variety of bacteria dwelling in, or spreading through, the rhizosphere or phyllosphere. Therefore, when dealing with disease processes that are being expressed in a host, which has a microbial population of its own as in the case of mushrooms, it is difficult to distinguish between diseases caused by a single pathogen and those requiring interactions with other microorganisms in the immediate environment.

Well known mushroom diseases such as brown blotch caused by *P. tolaasii* (Moquet *et al.*, 1996), and ISN caused by *E. americana* (Inglis *et al.*, 1996b), also require very high inocula (10^8 cells) for the initiation of respective diseases, under test conditions. Inglis *et al.* (1996) mentioned a variation in the intensity of disease symptoms by *E. americana* and reported an interaction between *P. fluorescens* and *E. americana* in the formation of ISN of mushrooms. They also suggested a contributory role played by *P. fluorescens* in the expression of disease symptoms. Moquet *et al.* (1996) in their study of blotch disease observed a variation in the susceptibility of *P. tolaasii* to different species of mushrooms tested and reported a variation in the intensity of disease expression amongst the different species of mushrooms tested. These variations were

perhaps a reflection of polymicrobial interactions between pathogenic and the non-pathogenic strains present in the sporocarps of different mushroom species. Initiation of Cavity disease expression involves an antagonism played by PRC120, a natural mushroom isolate. If natural antagonists like PRC120 were not present in nature, perhaps Cavity disease would be a very prevalent disease in mushroom industry.

In the first publication on Cavity disease by Gill and Cole (1992) a variation in development of disease symptoms from mild to severe tissue necrosis was reported. However, no plausible explanation for such a phenomenon was put forward by them. Gill and Cole's observation has been confirmed in the course of assays presented in this work and evidence is provided that the differential expression of disease symptoms could be explained in terms of the relative abundance of PRC120 in an individual sporocarp sample or be due to the production of an antibiotic by PRC120 that targets BG164R. This has been confirmed by *in vitro* and *in vivo* inhibition assays.

Brogden and Guthmiller (2003), in a recent review on polymicrobial diseases, suggested that future research should be directed towards elucidating mechanisms of microbial interaction and disease pathogenicity in order to understand the molecular mechanisms of disease formation (Brogden & Guthmiller, 2003). Based on the observations presented in case of Cavity disease initiation it is tempting to suggest that mushrooms could be used as a model to study 'Polymicrobial interactions' and previous studies on mushroom diseases should be re-visited in order to explore the possible roles played by the indigenous mushroom bacteria in the expression of those diseases.

BG164R has to overcome the initial inhibition by PRC120 prior to achieving successful establishment as a pathogen of mushrooms and, as a result, the inoculum has to be fairly high for expressing disease symptoms. Disease progression in nature starts from very few pathogenic cells acting as the inocula. The pathogen thereby goes through the stages of establishment and colonisation of the host tissue. Disease symptoms are displayed only after successful completion of the initial stages. This is, however, not observed in the case of BG164R infections starting from 20-24 cells. As demonstrated, high inoculum of BG164R is the absolute requirement for initiation of Cavity disease in mushrooms, which is in contrast to the data presented by Atkey *et al.* (1992), with the

type strain RR3 of *B. gladioli* pv *agaricicola*. They showed the initiation of soft rot disease expression within 72hr with a low inoculum of 3-4 cells. Moreover, with an inoculum of 1000 BG164R cells, mushroom samples exhibit disease symptoms after 36h incubation. With an inoculum as high as 10^6 cells, BG164R consistently produced Cavity disease symptoms within a significantly short time of 16hr, indicating that primary inoculum size plays a vital role in disease initiation. In the case of natural infections, initiation of Cavity disease would also be dependent on the presence of a high inoculum. Thus, chance factors involved in the presence of high inocula of pathogenic bacteria in nature and the presence of antagonists, like PRC120, are possible explanations for the sporadic occurrence of Cavity disease in the mushroom industry.

Cell density-dependent gene regulation or 'quorum sensing', identified initially as 'co-operative behavioural patterns' and exemplified by feeding and sporulation in myxobacteria and actinomycetes, swarming motility of *Vibrio parahaemolyticus* and *Proteus mirabilis* (Fuqua *et al.*, 1994) has often been associated with the regulation of virulence genes (Lewenza *et al.*, 1999; Anand & Griffiths, 2003; Jude *et al.*, 2003). These systems regulate the production of virulence factors like protease, lipase and siderophore in the different species, thereby playing an indirect role in the pathogenesis of the bacteria. Besides the regulation of virulence factors, a number of other functions such as conjugation mediated by the tumour-inducing Ti plasmid of *Agrobacterium*, bio-luminescence of *Vibrio fischeri*, swarming motility in *Serratia liquefaciens* and antibiotic production in *Erwinia carotovora*, have been reported to be under the regulation of quorum sensing signals (Fuqua & Greenberg, 1998). In *P. chlororaphis* (PCL1391), an important biocontrol strain of tomato foot and root rot disease caused by *Fusarium oxysporum*, the production of the antifungal compound, phenazine-1-carboxamide, has been demonstrated to be under the regulation of quorum sensing signals (Chin-A-Woeng *et al.*, 2001).

Pseudomonas aeruginosa has two quorum sensing systems, popularly known as the *las* (Pearson *et al.*, 1995) and the *rhl* (Ochsner & Reiser, 1995) are present. The former is involved in the productions of signalling molecules known as N-(3-oxododecanoyl)-L-homoserine lactone (HSL) and has been associated with the activation of a wide range of virulence genes and production of pyoverdine siderophores. The *rhl* system has N-

butyryl-L-HSL as the signalling molecule and is involved in the activation of rhamnolipid synthesis genes and *lasB* genes, besides production of some secondary metabolites like pyocyanin, cyanide and chitinase (Whiteley *et al.*, 1999). Chapon-Harve *et al.* (1997) have demonstrated that the type II secretion system of *Pseudomonas aeruginosa*, the xcp pathway, is involved in the production of virulence factors, and is under the control of both the *las* and the *rhl* quorum sensing systems (Chapon-Herve *et al.*, 1997). Perhaps in case of Cavity disease too, the *gsp* system, which has been demonstrated to be involved in symptom production, is under the control of the quorum sensing signals.

The quorum sensing systems identified in the different members of *Burkholderia*, are *LuxR* homologues and are known as *cepI-cepR* system in case of *B. cepacia* (Lewenza, 1999) and *bviI-bviR* system in case of *B. vietnamiensis* (Conway & Greenberg, 2002). These systems have been demonstrated to regulate the production of virulence factors like protease, lipase and siderophore production of the strain. Interestingly, McKenny *et al.* (1995) reported an inter-specific communication between *B. cepacia* 10661 and *P. aeruginosa* PAO1 in the production of virulence factors of *B. cepacia*. They offered evidence of an increased amount of protease, lipase and siderophores in a midlog phase culture of *B. cepacia* in the presence of concentrated extracts from culture supernatants of *P. aeruginosa* (McKenny *et al.*, 1995). In the absence of data on the effect of BG164R on sterile mushroom tissue, the possible role of the other endogenous mushroom bacteria in the generation of such inducing signals can also not be eliminated. As presented in section 5.3.3 and figure 5.2, there is a consistently high population of *P. fluorescence* strains in mushroom pieces, and their role, if any, in the expression of disease is unknown. Perhaps, Cavity disease is also an effect of interspecies signalling between BG164R and *P. fluorescence*. Assays with refined conditions to sterilise mushroom tissues in future should lead to the identification of any involvement of interactions between the non-pathogenic endogenous mushroom bacteria and BG164R in the induction of Cavity disease.

Chapter VI

Conclusions.

6.1. Introduction.

Burkholderia gladioli pv. *agaricola* BG164R, is a micro organism with the potential to cause severe disease on economically important crops such as the edible mushroom, *A. bisporus*. The objective of this research was to identify the molecular mechanism(s) by which BG164R expresses Cavity disease symptoms on button mushrooms. One of the key findings of this research is that the typeII secretion pathway is required for virulence of BG164R in the expression of a mushroom disease. The consistent isolation of GSP mutants, with a 'no-cavity' phenotype, in more than one mutagenesis screening strongly suggests that toxin(s)/protein(s) secreted by the type II secretion pathway lead to the expression of disease symptoms. Gill and Tsuneda's proposal (1997), that Cavity disease is a combined effect of chitinase, glucanase and toxins, is enhanced with the additional finding that a putative polyketide synthetase gene product may be involved in pathogenesis. Cavity disease requires a fairly high inoculum of the pathogen at the initiation of colonisation of the host, which provides a competitive advantage to the pathogen over the antagonistic microflora present in the mushroom. Such high density of inocula is not very frequently encountered by the button mushrooms in commercial farms, where all conditions for cultivation are controlled. Thus, the sporadic occurrence of the disease can be explained by the variable outcome of the (initial) competition between endogenous mushroom bacteria and the pathogen in the initial colonisation of the mushroom.

The main objective of this chapter will be to summarise the key findings, to outline models describing the steps involved in the formation of Cavity disease on mushrooms by BG164R and to discuss future directions of work on this project, including key tests of the models.

6.2. Summary of Results.

The project was initiated with two broad objectives with respect to Cavity disease formation. First, to identify the *Burkholderia gladioli* genes involved in degradation of the mushroom tissue. Second, to describe the stages through which the pathogen successfully establishes itself on the host and causes the symptoms of Cavity disease. The present section discusses the project achievements in relation to these objectives (as described in section 1.5).

Identification of BG164R genes involved in the expression of Cavity disease.

An efficient bioassay was required to screen for mutants that could no longer cause disease. The mushroom bioassay system developed in the course of this study has been used to successfully isolate avirulent (no-cavity) mutants. The majority of BG164R mutants isolated on the basis of this bioassay have mutations in the different genes required for formation of the GSP apparatus of *B. gladioli* pv. *agaricicola*. This suggests that the Cavity disease process is dependent on the secretion of tissue degrading enzyme(s) via this type II secretion system. It is also likely that Cavity disease is a multifactorial process and therefore avirulent mutants are likely to be isolated only when a common system involved in the secretion of all these factors is blocked. Therefore, the bioassay system used may not have allowed detection of mutants with reduced cavity forming phenotypes resulting from absence of a single factor involved in the disease process.

The assay also had other limitations: Firstly, mushroom tissues are neither sterile, nor colonised by identical populations of endogenous bacteria, which may result in variable disease outcomes. Secondly, transferring test bacteria with toothpicks created variability in the inoculum size, which might have masked isolation of mutants with reduced capacity to form cavities in the course of mutagenesis screenings. These factors need to be considered in any future attempts to isolate mutations in genes required for Cavity disease.

Development of the bioassay was followed by the identification of the genetic markers present naturally in BG164 and establishment of an efficient mutagenesis system. BG164 was found to be rifampicin-sensitive. For practical reasons, a spontaneous rifampicin resistant mutant, BG164R, was isolated. BG164R was judged to be as virulent as its parent strain BG164. The genes involved in the virulence of BG164R were interrupted using a mini-Tn5KmlacZ2 mutagenesis system designed to generate random insertion mutants, which were phenotypically screened for expression of disease symptoms on mushrooms. Genes interrupted were identified by sequencing of genomic DNA adjacent to the transposon insertion sites. Prior to screening for no-cavity phenotypes, random integration of the transposon into the genome of BG164R was tested by the isolation of auxotrophic mutants, which resulted in 1.5% auxotrophs suggesting the randomness of transposition. The efficiency of the transposon to randomly insert into genes of *B. gladioli* is confirmed by the fact that the mutants isolated had mutations in different GSP genes involved in the formation of type II secretin, and that an antifungal-reduced mutant was also isolated using this mini-Tn5KmlacZ2 mutagenesis system. Screening of 10,000 mutants for 'no-cavity phenotype' led to the isolation of 8 prototrophic GSP mutants. A second round of mutagenesis, in an attempt to isolate mutations outside the GSP region identified a putative polyketide synthetase gene as a candidate.

The GSP has been shown to be required for Cavity disease, suggesting that proteins and/or toxins secreted by the GSP are responsible for cavity symptoms. The mutations identified were in *gspE*, *gspK*, *gspD*, *gspF*, and the *gspL* genes of the type II secretion apparatus of *B. gladioli* pv. *agaricola*, which prevent secretion of the virulence factors required for forming cavities on mushrooms. In the course of this project, analysis of the DNA sequences of sub-clones from the cosmid able to complement GSP mutants led to the identification of four more homologues of GSP genes, partial DNA sequences of which have been presented as Appendix III.

A unique mutation in a gene that has significant sequence similarity to the polyketide synthetase (*pks*) gene of *Myxococcus xanthus* was also isolated. An interesting feature of this mutant is that the mutation is complemented by the *gsp* operon-containing cosmid. The mutant, unlike the *gsp* mutants, secretes protease(s) in spite of not being

able to cause cavities on mushrooms. As discussed in Chapter IV, this putative polyketide synthetase gene may have a dual function. Polyketide synthetases, in general, participate in non-ribosomal peptide synthesis and act as modular type I polyketide synthases. Polyketides have a diverse range of functions, toxins being an example. It was demonstrated that the *pks* mutant still retains the capacity to inhibit fungal mycelia; thereby suggesting that the compound assembled by an enzyme produced by translation of the gene is not involved in the inhibition of fungal mycelia. Since non-ribosomal peptide synthesis is one of the functions of polyketide synthetases, it is also possible that the enzyme assembles a unique peptide product that is secreted by the GSP of BG164R.

Colonisation of mushrooms and expression of disease by BG164R.

The final aim was to understand the host-pathogen relationships involved in the expression of Cavity disease. The strains comprising the endogenous microbial population present in the sporocarp of the mushroom have been identified. One of these endogenous strains, constantly present in the mushrooms, has been shown to be a natural antagonist of BG164R. The strain, PRC120, was isolated from mushrooms in New Zealand, for the first time. Other strains of this bacterium, identified as *Ewingella americana*, have been reported to be the causative agent of internal stipe necrosis of mushrooms. However, the PRC120 isolate does not show any signs of causing necrosis on mushroom. *In vitro* plate assays (lawn tests) confirmed results obtained from *in vivo* assays on mushroom slices in which mixed populations of BG164R and PRC120 clearly indicated inhibition of BG164. This may explain why the disease occurs sporadically, as natural inocula of the pathogen, BG164, may often be too low to overcome this inhibition and would therefore fail to colonise the mushroom tissues.

Once the pathogen establishes itself on the host, it multiplies over a time and cause disease only when a certain quorum of cells is reached, as demonstrated in chapter V. Thus, expression of Cavity disease also appears to be dependent on a certain density of pathogenic cells on the mushroom tissues. Quorum sensing could lead to the expression of Cavity disease by:

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- a) Density-dependent induction of the *gsp* gene cluster resulting in the secretion of products, which cause Cavity disease.
 - b) Induction of virulence genes, products of which are directly involved in the rapid degradation of the mushroom tissue.

Tagging candidate genes with reporter genes like *lux* or *lac* systems and monitoring the expression of the reporter at lower concentrations of bacteria in the presence of culture extracts from spent cultures could be used to test these hypotheses.

6.3. Models for Cavity disease formation by BG164R.

The findings presented can be discussed in the context of three different models of Cavity disease process. In all models the mushroom sporocarps must be inoculated with a population of *B. gladioli* pv. *agaricicola* (BG164R) greater than 10^2 cells in order to overcome inhibition by the local flora, such as PRC120. This is the first step common to all three models after which each model is based on three distinct pathways leading to Cavity formation. It is worth noting that these possibilities are not mutually exclusive.

Common Steps involved in the initiation of disease.

- Inoculation of mushroom sporocarps with a population of pathogenic BG164 cells capable of out-competing endogenous PRC120 populations.
 - Competition of BG164 with PRC120 and the establishment of the BG164 population on the mushroom sporocarps as the dominant population.
-

Model I: Quorum sensing-dependent formation of type II secretin.

- At a threshold concentration of cells, *gsp* genes are induced and BG164 produces a functional type II secretin.
- Induction of tissue degrading enzymes by a polyketide derivative acting as an auto inducer.
- Formation of cavity/degradation of mushrooms.

Model II: Secretion of the polyketide derivative and tissue degrading enzymes by GSP of *Burkholderia gladioli*.

- Tissue-degrading enzyme(s) are induced by a quorum sensing mechanism when BG164R cells reach a certain threshold concentration.
- Induction and secretion of both the tissue degrading enzymes and polyketide derived toxin(s) by GSP of BG164R.
- Degradation of mushroom sporocarps by a combined action of all compounds.
- Formation of cavity/degradation of mushrooms.

Model III: Quorum sensing-dependent activation of the polyketide synthetase gene.

- Polyketide synthetase gene induced by a quorum sensing mechanism when BG164R cells reach a certain threshold concentration.
 - The polyketide synthetase enzyme assembles a toxin, which ruptures the hyphal cell walls of the mushroom sporocarps leading to the exudation of cytoplasmic contents from ruptured fungal mycelia.
-

-
- Induction of the genes responsible for translation of the tissue degrading enzymes of BG164R by compound(s) present in exudates liberated by the damaged mushroom mycelia.
 - The GSP of BG164R secretes one or all the mushroom degrading enzymes.
 - Formation of cavity/degradation of mushrooms.

6.4. Testing the models.

Firstly, a bioassay less susceptible to variation would be an advantage for all aspects of the investigation of Cavity disease. One possibility would be to aseptically culture mushrooms for use in the bioassay. Once the bioassay conditions have been re-standardised, further mutagenesis could be carried out in an attempt to identify additional genes involved in Cavity disease.

Another important issue to be addressed is the isolation and identification of products that are secreted by the GSP of BG164R and their role in the formation of Cavity disease process. The secretory mutants isolated during the course of this study are likely to be of use in the identification of the active components that take part in the degradation of the mushroom tissues.

Testing Model I:

The first point to resolve is the chemical nature of quorum sensing signals to which BG164R responds in the formation of Cavity disease. If homoserine lactones, the common auto inducers in Gram-negative bacteria are involved, they can be detected by HPLC analysis of culture supernatants from spent cultures. Growing fresh BG164R cells in presence of extracts from spent cultures could induce the production of active compounds at a much earlier stage of growth. Thus, isolation of active tissue degrading compounds from supernatants of young, actively growing cultures of BG164R grown in

presence of quorum signalling molecules extracted from spent cultures would indicate whether quorum sensing is involved in the disease process.

To identify whether quorum sensing activates *gsp* genes specifically, the expression of a reporter gene-tagged *gsp* mutant at various phases of growth in the presence and absence of extracts from spent culture supernatants and quorum signalling molecules could be monitored. Several mini-Tn5*KmlacZ*-generated *gsp* mutants isolated show active translational fusions to the promoter-less *lacZ*, which could be exploited in such assays.

Testing Model II:

A confirmed observation is the indirect involvement of a product produced by the putative polyketide synthetase in the expression of cavity disease, as mutants lose the capacity to form cavities on mushrooms. It is postulated that the secretion of the product(s) assembled by an enzyme produced by the putative polyketide synthetase gene is dependent on the GSP of *Burkholderia gladioli*.

An interesting characteristic of Cavity disease, first reported here, is the ability of pCosGSP to complement the putative polyketide synthetase mutant, BGII-107. It is predicted that the 5kb DNA downstream of the *gsp* region in the cosmid harbours genes able to complement the mutation in BGII-107. A strain of BG164R with mutations in both the *gsp* operon and in the putative polyketide synthetase gene would allow testing of this hypothesis. Also required would be a cosmid sub-clone of the 5kb DNA region containing the putative polyketide synthetase gene. Effects of such a double mutant strain on the mushroom slice in the presence of a sub clone of the cosmid polyketide synthase gene would help us to confirm the association of GSP in the secretion of product assembled by the enzyme produced by the putative polyketide synthetase gene.

Testing Model III:

The *lacZ* reporter gene in the polyketide synthetase mutant, BGII-107, also forms an active translational fusion. The *lacZ* reporter gene can be used in assays as detailed above to test the quorum sensing-dependent activation of this gene.

On the basis of DNA sequence similarity, it has been concluded that the mutation in BGII-107 is in a putative polyketide synthetase gene. Firstly, the full sequence of the genomic DNA flanking the transposon insertion needs to be determined in order to locate the exact position in which the transposon has inserted. This would establish the authenticity of the mutation being in the putative polyketide synthetase gene. Sequencing using primers designed to read out from the ends of the transposon into the chromosomal DNA or, alternatively, primers based on the known DNA sequence could, be used to identify the transposon insertion point.

To test the stepwise action of the toxin and the degradative enzymes, the individual active components need to be identified and, at least partially, purified. Effects of challenging sterile mushroom mycelia with the individual components and with various combinations of components to an actively growing culture of wild type BG164R, should enable determination of specific auto inducers required for the activation of genes.

6.5. Concluding remarks.

Answers to the questions raised in this chapter should allow adoption of an appropriate model for the Cavity disease process. This project has provided major insights into the process of the rapid mushroom tissue degradation by BG164R. In addition the basic biology and genetics of BG164R have been characterised, and methods for genetic manipulation of *Burkholderia gladioli* pv. *agaricola* (BG164R) have been established in the course of this project.

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*Appendix I***MEDIA, ANTIBIOTICS AND SUPPLEMENTS****(A) MEDIA:**

All media were made up in dH₂O and autoclaved at 121°C for 20 minutes at 120kPa

1. Luria Bertani media (LB). (Miller 1972)

1% w/v Bactotryptone

0.5% w/v Yeast extract

0.5% NaCl

pH 7.0

2. LB Agar

LB media with 1.5% w/v agar

3. M63 Minimal media. (Miller 1972)

10X M63 salt stock solution(autoclaved separately)

1M KH₂PO₄

0.15M (NH₄)SO₄

0.018mM FeSO₄.7H₂O

pH 7.0 with KOH pellets.

M63 media.

1XM63 media made by 1:10 dilution of 10XM63 stock salts in sterile dH₂O, followed by addition of sterilised 100 times concentrated stock solutions and made up to a final concentration of:

0.02% MgSO₄

0.0005% Thiamine

0.2% Glucose

4. M63 Minimal Agar.

1XM63 media supplemented with 1.5% w/v agar

5. Nutrient Agar.

0.8% w/v Nutrient broth powder (GIBCO BRL)

1.5% w/v agar.

6. Kings 'B' Media. (King *et al.* 1954)

2% w/v Bactotryptone
65mM K₂HPO₄ anhydrous
60mM MgSO₄·7H₂O
1.5% v/v Glycerol

7. SOC Media (for electroporation). (Sambrook *et al.* 1989)

2% w/v Bactotryptone
0.5% w/v Yeast Extract
10mM NaCl
2.5mM KCl
10mM MgCl₂
20mM Glucose

8. Potato Dextrose Agar.

PDA Oxoid as per manufacturer's instruction
3.9g/L of dH₂O

9. Motility Agar.

0.1% w/v Bactotryptone
0.05% w/v Yeast Extract
0.5% w/v NaCl
0.3% w/v Agar

10. Milk Agar (Protease plates).

Solution A: 1% skimmed milk solution
Solution B: 0.8% Nutrient Broth Powder
1.5% Agar

The solutions were separately sterilised, cooled to about 55°C and mixed in a proportion so as to make a final concentration of 0.1% skimmed milk solution in 0.8% Nutrient broth agar.

11. Water Agar.

1.5% w/v agar in distilled water.

12. Chitin Agar.

1% Chitin extract mixed in equal volumes with 2X Nutrient Agar.
10mL overlayed onto pre-made Nutrient agar plates.

Preparation of 1% Chitin extract.

- (a) 5g Chitin dissolved in 200mls of 85% Phosphoric acid for 72hr at 4°C.
- (b) Dissolved chitin was washed three times in water by adding 2.5L water, followed by stirring and precipitating chitin each time.
- (c) The pH of the dissolved chitin was adjusted to 6.8 with NaOH.
- (d) Washed three times with water.
- (e) After the final wash, chitin was precipitated by centrifugation and resuspended in water to make a final solution of 1%.
- (f) Autoclaved and stored at 4°C.

13. Compost Malt extract agar.

0.75% Malt extract (Oxoid)

1.5% Agar

100mL Sterile Compost Infusate.

pH adjusted to 7.4 with 1(N) NaOH.

Autoclaved .

Preparation of Sterile Compost Infusate.

- (a) 5% w/v oven dried commercially prepared mushroom compost left to infuse in water for 1hr.
- (b) Temperature was raised to 100°C for 5min to remove microbial matrix and stirred vigorously.
- (c) The mixture was left aside for further 2hr, cooled and filtered through four layers of muslin cloth.
- (d) Autoclaved twice.

14. Mushroom juice extract media.

10% w/v Fresh mushroom sporocarp tissue from young mushrooms macerated in sterile distilled, decanted through nappy liner and autoclaved immediately.

15. Mushroom juice extract Agar media.

Mushroom juice extract media supplemented with 1.5% w/v agar.

(B) ANTIBIOTICS AND SUPPLEMENTS:

The following concentration of antibiotics and supplements were added to bacterial cultures on Agar plates or broths, unless otherwise stated, either to maintain selective pressure or to identify strains and constructs.

Ampicillin	(Ap)	100µg/mL in glass dH ₂ O
Chloramphenicol	(Cm)	15µg/mL in 100% Ethanol
Gentamicin	(Gm)	30µg/mL in glass dH ₂ O
Kanamycin	(Km)	50µg/mL in glass dH ₂ O
Nalidixic acid	(Nal)	30µg/mL in 0.05M KOH solution
Streptomycin	(Strep)	50µg/mL in glass dH ₂ O
Tetracycline	(Tet)	15µg/mL in 100% Methanol
Rifampicin	(Rif)	50µg/mL in Dimethylsulfoxide
5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside	(Xgal)	25µg/mL in Dimethylformamide

*Appendix II***BUFFERS AND REAGENTS****I. GENERAL BUFFERS AND SOLUTIONS****T₁₀E₁ (TE)**

10Mm Tris- HCl

1mM EDTA

In dH₂O, pH adjusted to 8.0**TAE**

50mM Tris base

1 mM EDTA

0.11% v/v Glacial acetic acid

In dH₂O, pH adjusted to 8.0**DNA loading buffer for agarose gel electrophoresis**

30% glycerol

0.25% Bromophenol blue

0.25% Xylene cyanol FF

10 μ .ml⁻¹ RNAase**II. SPECIFIC BUFFERS AND SOLUTIONS****Alkaline Lysis Preparation of Plasmid/Cosmid DNA****Solution I** (Stored at 4°C)

1% w/v glucose

25mM Tris- HCl, pH 8.0

10Mm EDTA

Solution II (prepared fresh)

1% SDS

0.2N NaOH

Solution III (stored at 4°C)

3M Potassium acetate

8.7% v/v Glacial acetic acid

Genomic Preparation of DNA

GES solution

5M Guanidium thiocyanate

100mM EDTA, pH 8.0

20% v/v dH₂O

Heat at 65°C until dissolved.

Add 0.5% N-laurylsarcosine (from 10% solution).

Make up to volume with dH₂O and filter through 0.45µm filter

Silver Staining Solutions for Protein Gel Electrophoresis

Wash Solution I

50% Methanol

10% Acetic Acid

40% dH₂O

Wash solution II

10% Gluteraldehyde

90% dH₂O

Staining Solution

Reagents added according to the order presented with continuous stirring. If at any stage the colour of the solution went brown the solution was titrated back by adding NH₄OH drop wise until clear.

0.3% NaOH	15.3mL
Concentrated NH ₄ OH	1.03mL
0.2g/mL stock of AgNO ₃ in H ₂ O	3mL
100% Ethanol	55mL

Developing Solution

1% Ethanol

0.25% Citric Acid solution (1%)

0.025% Formaldehyde (38%)

98.725% dH₂O

Fixing Solution

5% Acetic Acid solution

95% dH₂O

Lysis buffer for Protein sample

2% SDS
1M Tris (pH 6.8)
4% β -mercaptoethanol
0.05% Bromophenol Blue
10% Glycerol

Solutions for Southern and Colony Hybridization**Depurination solution (DNA blotting)**

0.25N HCl

Denaturation solution (DNA blotting) (Freshly prepared)

0.4N NaOH

20 x SSC (stored at 4°C)

3M NaCl
0.3M Na₃Citrate
In dH₂O, pH adjusted to 7.0

Denaturing Solution (Colony Hybridisation)(Freshly prepared)

0.8g NaOH
28mL dH₂O
12mL NaCl solution (5M)

Neutralisation Solution (Colony Hybridisation)(Freshly prepared)

40ml Tris-HCl (1M)
24mL NaCl solution (5M)
16mL dH₂O

Solution to remove excess debris in Colony hybridisation

3XSSC
0.1% SDS

Pre-Hybridization buffer

9mL SSC
1.5mL Denhardt's solution
1.5mL SDS (10%)
0.15mg Blocking reagent
18mL dH₂O

Hybridization buffer

Pre-hybridisation buffer with 200Kcpm (per 30mL buffer) labelled probe

Low stringency wash buffer

0.1% SDS

2x SSC

Medium stringency wash buffer

1x SSC

0.1%SDS

High stringency wash buffer

0.1% SDS

0.1x SSC

Sequencing**10x TBE**

890mM Tris base

890Mm Boric acid

20Mm Na₂EDTA.2H₂O

Sequencing Gels

31.5g Urea

36ml dH₂O

6ml 50% Long Ranger Gel solution

9ml 10x TBE

500µl 10% APS (made fresh)

50µl TEMED

*Appendix III***PRIMERS USED AND DNA SEQUENCES****A. Primers used.**

T7 promoter 5' GTAATACGACTCACTATAGGGC 3'

T3 promoter 5' AATTAACCCTCACTAAAGGG 3'

B. Sequences flanking transposon insertion points.**1) pSPRC12, T7 end.**

CGGCTCGGTGCCGAACACGATGCGATGCACTTCGGTGTCGGAGCCGGGATAGTCGATGG
TCGCGCCGGTCACGCCGCCGTCCCAGTTGCGCGGCCGGAACAGGTCGTGCGCASCSCGC
CAGCGTCCTCGCCGCGCACGTCTGAAGCGGAACGCGTTGTCGGTGGGCTGCCAGGCGATC
GGCCGCGCGCGCACCTGCSTCGTCGCCGGCGCTCTCGAGCAGCAGCGCCACTCGCTGGG
CTTCCTCGCGCAGGTTCGGTGCGATTGCGATGCATGGTCAGCGTGGCCGCCGACACGAGG
ATCCCCGCGATCACGAGCACGACGAGCATCTCGAGCAACGTGAAGCCACGCATCCGCCG
GGGSAAACGCAGCGAGGTTCGTGCCGGTGGTTTCGCATGTCAAACGAACTGACCAAGAGG
GAAGGGACGGGCGGTGCGATCCGATGCGCCGGGCTTACTGCCAGGAACCGATGTCGGCG
TCGTTGTTCTCGCCGCCTTCCTTGGCGTCGGCGCGTAGCTGAACACGTCGATCTCGCCG
TGCACGCCCGGGTTTCAAGTACTTGTACGGGTTGCCCCAGGGATCGTTGGGCAGGCGCTC
GAGGTAGCCGCCGTCTTCCAGTTGTTTCGGCACCGGATCGGTGGTGGGCTTGGCGATCA
CGATTGCAGGCCCTGCTCCTGGGTTCGATAGCGGCCGTTGTCGAGGCGGTAGAGCTTGA

2) pSPRC12, T3 end.

GGCAGCTGCGACCGTGGTGCGGCCCAAGAAGGCCTGCACGGCGCGCTGATCTCGCGGA
TCAAGATCATGGCGCACGACATCGCCGAGAAACGCCTGCCGCAGATGGCCGGATCACGC
TGCGGCGGCGCCGGTGGACGTGCGGGTCTCGACGCTGCCACCGGCCACGCGAGCGCGC
GGTGCTGCGTCTGCTGGAAAAGGATGCGCGCCTGAACCTCGAGGCGCTCGGCATGGGCC
GACACGCTGGTGCAGTTTGACAAGCTGATCGGCCGCCCGCACGGCATCGTGTGGTCAAC
GGCCCCGACCGCTCGGAAGACCACACGCTCTACGCGGCGATGTCGCGCTGGAGACGGCC
ACCACCAACATCATGACGGTTCGAGGATCCGATCGAATACTACCTGTCCGGCATCGGCCA
GACGCAGGTGAACGAGCGGATCGGCATGAGCTTCGCGCGCGCTGTCTCGATCCTGCGCC
AGGTCCGGACGTTTCATATCGGCGAAATCCGCGATCTGGAAACCGCGCAGATCGCCGTG
CAGCTCGCT

3) pSPRC40, T7 end.

CGTGCTGTGCGACAACGGCGAGATCATCGTGCTCGGCGGCCTGATGCAGGACAACCTACC
AGGTCAGCAACAACAAGGTGCGCTGCTCGSGACATCCCGTGGATCGGCCAGTTGTTCCG
TTCGGAAGGCAAGCAGCGCCAAGACCAACCTGATGGTGTTCCCTGCGCCCGGTGATCCTC
AGCGACCAGGCCACYACCCAGGCCGTCACCGCGAACCGCTACGACTACATCAGGGCGTG
CAGGGCGCGTACAAGTCCGACAACAACCTGATCGCGACAAGGACGACCCGGTGCGCGCG
CGATGCCGCTYGGGCCGACAGGGCGGGCGCGGCCGCGAACCTGTTTCGACCTGACGCGCA
TGACGCACCCCGTTATCGGCCCTCTCGCAGCGCACCGACCAACGTGCCGGCTCCCGCG
GTGGCCAC

4) pSPRC40, T3 end.

CTCGCCTTCCACCTCGCCGACCACCTGCGCCGCGCTGCCGTCTTGGCGCATAGGGTGTT
GATGCTGACTCTTATACACAAGTGCGGCCCGCCGCGTTTAATGACCAGCACAGTCGTGAT
GGCAAGGTCAGAAATAGCGCTTAGGTCTGCTCGTGAAGAAGTGTTGCTGACTCATACCAG
GCCTGAATGCCCCATCATCCAGCCAGAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTG
TTGTAGGTGGACCAGTTGGTGATTTTAACTTTTGGCTTTGCCACGGAACGGTCTGCGTTG
TCGGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAAGTTTCGATTTATTCAACAAAG
CCGCCGTCCCGTCAAGTCAGCGTAATGTCTCCAGTGTTACAACCAATTACCAATTCTGA
TTAGAAAACTCATCGAGCATAAA

5) pSPRC88, T7 end.

TCCTGCTCGACGTCAACGGCGTGACGCCGAGATCGTCGAGAAGCTGCGGCCCTTCGTGA
CGGTGCTGCCGTCCACCACGCCGGTCAACCTCAATACTGTCTCTTGATCAGATCTGGCC
GCCTACGAATTCCCGGGGATCCCGTCGTTTTACAACGTCGTGCTGGGAAAACCCTGGCG
TTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTCCAGCTGGCTAATAGCGAAGAG
GCCCCGACCGATCGCCCTTCCCAACAGTTGCGCACGCTGAATGGCGAATGGCGCTTTGC
CTGGTCCGGCACCAGAAGCGGTGGATAGCTGGCTGGAGTGCGATCTTCCTGAGGCCGAT
ACTGTCGTGCTCCCCTCAAACCTGGCAGATGCACGGTTACGATGCGCCCATCTACACCAA
CGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCACCGGAGAATCCGACGGGTT
GTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGAATT
ATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTGCGTTA
CGGCCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAG
AAAACCGCCTCGGGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCAGGAT
ATGTGGCGGATGAGCGGCATTTCCGTGACGTCTCGTTGCTGCATAAACCGACTACACA
AATCAGCGATTTCCATGTTGCCACTCGCTTTAATGATGATTTTCAGCCGCGCTGTACTGG
AGGCTGAAGTTCAGATGTGCGGCGAGT

6) pSPRC88, T3 end.

GAGCGCGAACGACACGCATTGCACTCGGCCAGCCGGCGGTTTCGCGCGGGCAAAACAACA
ATCAACGTGCTCAAGAGCTCCTCTCCCGGATGGCGTTGTTATAGCTGATCGGAACCCGG
ACCACGCGCGTGTGTGCGTGTGGGGTCACGATACACGAGCGAGGTGCGGTCGATTTCC
GCGCGCCCGTGCTCAATATGACCGTGAACGATGAAATAACTGGACGTCACGTCGATCTG
GGTCGGGTCCAGCAGCGTGTTCGGCACSCGGTGGCCTGCAGCGGACTGCATCGCCGAC
GTTGCGGAAGAACACGTTCTCGCGTCGCCACCAGCGTCTGCGCGGAGGACACCGAGAGC
CCCGGCAGCAGCGCGCGATCACCTCGGCGCGCGGTATTGAGGCTGACTCTTATACACAA
GTGCGGCCCGCCGCTTTAATGACCAGCACAGTCGTGATGGCAAGGTGAGAATAGCGCTG
AGGTCTGCCTCGTGAAGAAGGTGTTGCTGACTCATACCAGGCCTGATGCCCCATCATCC
AGCCAGAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGT
GATTTTGAACCTTTTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAAGATGCGTGATCT
GATCCTTCAACTCAGCAAAAGTTGATTTATTCAACAAAGCCGCGTCCCGTCAAGTCA
GCGTAATGCTCTGCAGTGTTACAACCAATTAACCAATTCTGATTAGAAAACTCATCGA
GCATCAAATGAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTT

7) pSPRC87, T7 end.

ACCACCGAGGAATCCTCGTGTGAGATCTGCAGCTTCAGGATGCCGCCCTCGGTGATCTG
CGGCTTGACGTGCAGGGTCAGGCCGACGTCTTGCGGTGCAAGGTATTGAAGGCGTTGC
TGTTGCTGCCGCTGGTCAGGTTGGCATAACGAACCGGTCGCGATCGGCACGTTCTGGCCC
ACCACGATCTTCGCTTCCTCGTTGTCGAGCGTGACCAGGTTAGGCGTGGACAGCACGTT
CGCGTCGGTGTTGCCGAGAAGGCCTGCAGCAGGGCGCCAGGCCAGTGATGCCGAACA
TGTTCTTGAGCCAGCCGATGTTTCAGGCCCGGCGTCAGCGCCCTTTGGCATAAGCGTGCG
CGTTTGGTGGAATAAAAGGGA

8) pSPRC87, T3 end.

ACCACCGAGGAATCCTCGGTAGATCTGCAGCTTCAGGATGCCGCCCTCGGTGATCTGCG
GCTTGACGTGCAGGGTCAGGCCGACGTCTTGCGGTGCAAGGTATTGAAGGCGTTGCTG
TTGCTGCCGCTGGTCWGGTTTTTCATACGAACCGGTCGCGATCGGCACGTTCTGGCCCAC
CACGATCTTCGCTTCCTCGTTGTCGAGCGTGACCAGGTTAGGCGTGGACAGCACGTTCTG
CGTCGGTNGTGCCCCGAGAA

9) pSPRC787, T7 end.

CGTGCTGTGCGACAACGGCGAGATCATCGTGCTCGGCGGCCTGATGCAGGACAACCTACC
AGGTCAGCAACAACAAGGTGCCGCTGCTCGGCGACATCCCGTGATCGGCCAGTTGTTT
CGTTCGGAAGGCAAGCAGCGCGCCAAGACCAACCTGATGGTGTTCCTGCGCCCGGTGAT
CCTCAGCGACCAGGCCACCACCCAGGCCGTACCGCGAACCCTACGACTACATCCAGG

GCGTGCGAGGGCGCGGTACAAGTCCGACAACAACCTGATCCGCGACAAGGACGACCGGTGG
CGCCGCGATGCCGCTCGGGCCGAGCCAGGGCGGGCGGGCCGCGAACCTGTTGACCTG
ACGCGCATGACGCACCCCCGGTTATCCGCCGCGCCGCAGCGCACCGACCAACGTGCCGG
CTCCCGCGGTGGCCACGCGACGGTGAACCCCGCGCCGATGCCCCGCGCCCGCCGTACCA
CGAGGTGCCGACGCCTGGCCTGACCAGCCAGCCCGGAGCGCACGTGAACGAGCCCGTGC
GCGATGCAGCGACCAGCGAGGGGCCGTGCGATCGCTGCTCGCCTGCTGCCCTACGGCTT
CGCAAGGCCGCGCCAGATCCTGGTTCGCGCAAGGACGGCGAGGCGATCGAGGTCTGGATCA
GCGATCGCAACCGACGCCGCGCTGGCCGAGGTGGCACGCAACTTCGGCGCGTTGANCGC
CGGCTGCGCCGACGAGCTCGCGCAGGCCATCAACAC

10) pSPRC787, T3 end.

CTCGCCTTCCACCTCGCCGACCACCTGCGCCGCGCTGCCGTCCTGGCGCATAGGGGTGT
TGATGCTGACTCTTATACACAAGTGCGGCCGCGCGTTTAATGACCAGCACAGTCGTGA
TGGCAAGGTCAGAATAGCGCTGAGGTCTGCCTCGTGAAGAAGGTGTTGCTGACTCATA
CAGGCCTGATCGCCCCATCATCCAGCCAGAAAGTGAGGGAGCCACGGTTGATGAGAGCT
TTGTTGTAGGTGGACCAGTTGGTGATTTTGAACCTTTCTTTGCCACGGAATCTGCGTTG
TCGGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAAGTTTCGATTTATTCAACAAAG
CCGCCGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTGTTACAACCAATTAACCAATTC
TGATTAGAAAACTCATCGAGCATCAAATGAACTGCAATTTATTTCATATCAGGATTAT
CAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAG
TTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAAT
ACAACCTATTAATTTCCCTCGTCAAAAATAAGGTATCAAGTGAGAAATCACCATGAG
TGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCA
ACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAACCGTTATTTCAT
TCGTGATTGCGCCTG

11) pSPRC147, T7 end.

TCCTGCTCGACGTCAACGGCGTGACGCCGAGATCGTCGAGAAGCTGCGGCCCTTCGGT
GACGGTGCTGCCGTCCACCACGCCGGTCAACCTCAATACCTGTCTCTTGATCAGATCTG
GCCGCCTAGGCGGAATTCCCAGGGAGTCCCGTCGTTTTACAACGTCGTGACTAGGAAAT
CCCTGGYGTTACCCAACCTAA

12) pSPRC147, T3 end.

AGCGCGAACGACAGGCGCATTGCACTCGSCAGCGRCGGCSTTCGCGCGGGGCAAAACAA
CAATCAACGTGCTCAAGAGCTCCTCTCCCGGATGGCGTTGTTATAGCTGATCGGAATCC
CGGGGACCACGCGCGTGTGTGCGTGTGGGGTCACGATACACGAGCGAGGTGCGGTGAT
TTCCGCGCGCCCGTGCTCAATATGACCGTGAACGATGAAATAACTGGACGTCACGTGCA
TCTGGGTGCGGTCCAGCAGCGTGTTCGGCATCGCCGACGTTGCGGAAGAACACGTTCTC
GCGTCGGCCACCAGCGTCTCGGAGGACACCGAGAGCCCCGCAGCAGCGCGGCGATCACC

TCGGCGCGCGCNGTATTTACTGACTCTTATACACAATCGTCTNTCGTTTAAATGACACAC
ATTGTTATGGCAATCAGATATTTTGAGTCTCTCCTCAAGAACTCTTTTGACTCATACCA
GTCTATCCCCATCATCCATCCAGAAATATAACCACTTGATGATACTTTTTTATGACCAT
TCGTATTTTAACTTTTTGTTTTACGGAACGTCTTTTCGGGAAGACTGATCTATTTCAAC
TTAAAAAATTCATTTATTTAACAAAGGCGCCC

13) pSPRC105, T7 end.

CTGTCGCGGGCTGATGCAGGACATCCCGGAAGTCGAGGACCTGCTGGAATCGGAGGACGA
CGCGCCGATCATCCGCATGATCAACGCCCTGCTCAGGAGGCGCGCGGAAGGCTTCC
GACATCCACATCGAGCCCTTCGAGAACGCCTCGGTCTGTCTCTTGATCAGATCTGGCCG
CCTACGAATTCCCGGGGATCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCG
TTACCCAACTTAATCGCCTTGACGACATCCCCCTTTCCAGCTGGTAATAGCGAAGAGG
CCCGCACCGATCGCCCTTCCCAACAGTTGCGCGCTGAATGGCGAATGGCGCTTTGCCTG
GTTTCCGGCACCAGAAGCGGTGAGCTGGCTGGAGTGCGATCTTCCTGAGGCCGATACTG
TCGTCGTCCCCTCAAACCTGGCAGATGCACGGTTACGATGCGCCCATCTACACCAACGTG
ACCTATCCCATTACGGTCAATCCGCCGTTTGTTCACGAGAAATCCGACGGGTTGTTA
CTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGAATTATTT
TTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTGCGTTACGGC
CAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTTACGCGCCGGAGAAAA
CCGCCTCGGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCAGGATATGTG
GCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACTACACAATCAG
CGATTTCCATGTTGCCACTCGCTTTAATGATGATTTTACGCCGCGCTGTACTGGAGCTGA
AGTTCAGATGTGCGGCGAGTTGCGTGACTACCTACGGTA

14) pSPRC105, T3 end.

GCGTCAGCCACCGAGGCGCTGACTCTTATACACAAGTGCGGCCGCCGCGTTTAAATGACC
AGCACAGTCGTGATGGCAAGGTCAGAATAGCGCTGAGGTCTGCCTCGTGAAGAAGGTGT
TGCTGACTCATACCAGGCCTGATCGCCCCATCATCCAGCCAGAAAGTGAGGGAGCCACG
GTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGTGATTTTGAACTTTTGCTTTGCCA
CGGAACGGTCTGCGTTGTGCGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAAAGTT
CGATTTATTCAACAAAGCCGCCGTCCCGTCAAGTCACGTAATGCTCTGCCAGTGTTACA
ACCAATTAACCAATTCTGATTAGAAAACTCaTCGAGCATCAAATGAACTGCAATTTA
TTCATATCAGGATTATCAATACCATATTTTTGAMAAGCCGTTTCTGTAATGAAGGAGAA
AACTCACCGAGGCAGTTCCATAGGATGGCAAGCATCCTGGTATCGGTCTGCGCATTCGG
ACTCGTCCAACATCAATACAACCTATTAATTTCCCTCGTCAAAAATAGGTATCAAGT
GAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTT
TTTCCAGACTTGTTCAACAGGCCAGCCATTAGCTCGTCATCAAAATCACTCGCATCAAA
CAAACCGTTATTCAATTCGTGATTGCG

15) pSPRC106, T7 end.

TCTCTGCTCGACGTCAACGGCGTGACGCCGAGATCGTCGAGAAGCTGCGGCCCTTCGTG
ACGGTGCTGCCGTCCACCACGCCGGTCAACCTCAATACCTGTCTCTTGATCAGATCTGG
CCGCCTACGAATTCCCGGGGATCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTG
GCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCCAGCTGTAATACGAAGAG
CCCGCACCGATGCCCTTCCCAACAGTTGCGCAGCTGAATGGCGAATGGGCTTTSCCTGG
TTTCCGGCACCAGAAGCGGTGGTAGCTGGCTGGAGTGCATCTTCCTGAGGCCGATACT
GTCGTCTGTCCTCCCTCAAACCTGGCAGATGCACGGTTACGATGCGCCCATCTACACCAACGT
GACCTATCCATTACGGTCAATCCGCCGTTTGTTCACGGAGAATCCGACGGGTGTGTT
ACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGAATTATT
TTTGATGGCGTTAACTCGGCCGTTTCATCTGTGGTGCAACGGGCGCTGGGTTCGGTTACGG
CCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAAA
ACCGCCTC

16) pSPRC106, T3 end.

GAGCGCGACACAGTCGCATTGCACTCGCAGCCAGCGGCSGTTTCGCTGCGGCCAAAACAAC
AATCAACGTGCTCAAGAGCTCCTCTCCCGGATGGCGTTGTTATAGCTGATCGGAACCCG
GACCACGCGCGTGTGTGCGTGTGGGGTCACGATACACGAGCGAGGTGCGGTTCGATTTCC
GCGCGCCCGTGCTCAATATGACCGTGAACGATGAAATAACTGGACGTCACGTCGATCTG
GGTCGGGTCCAGCAGCGTGTTCGGCACSCGGTGCTGCAGCGCGACTCATCGCCGACGTT
GCGGAAGAACACGTTCTCGCGTCGCCCACCAGCGTCTGCGCGAGGACACCGAGAGCCCC
GGCAGCAGCGCGCGATCACCTCGGCGCGCGTATTGAGGCTGACTCTTATACACAAGTGC
GGCCGCGCGTTTTAATGACCAGCACAGTCGTGATGGCAAGGTCAGAATAGCGCTGAGGT
CTGCCTCGTGAAGAAGGTGTTGCTGACTCATACCAWGCTGAWHTCCCCATCATCCAGC
CAGAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGTGAT
TTTAACTTTTCTTTGCCACGGAAC

17) pSPRC107, T7 end.

GGGGCGCTTGCGCGCAGCTCTCGSCGCSGGCGCGTGCGCAGACGGGCGATCGGCCATCG
TCTCGCGATGSTCTCGACCAGATAAGCCGTGAGCGCCGCCAGCGTCGGATGCTCGAAGA
ACACCGTCGGCATCAGATCCAGTTTCGTAGGTGCGATTCAATTCGGTGGCCAGTTCAGTC
AGCGAGATCGAGTCGAAGCCATAACCGGCCAGCTCCTCGTCGAGATCGATGTCCTCGGG
CTTCACCTTGATCAAGGCAGATACGCGGCTCAAGAGCGCCGCTCCGACCACGCGCGACA
GATCAACCCCGGCACCGGCGCTGCTGCGGGCGGCACGACGGCTTCGGTTCGTGTCTTCGTC
GGCCTGGTCCCCGGCCGTGCAACAGCGCGAAGCTTCTCGCGTCGCCCCGCCGCCACCAG
CACCTGCGCCGCATCGCCGTCACCGCCTGCGCGAAGCCGCCAGTGCCGCCCTCGTTCT
CCAGCGGCACCAGCCCGCGGTGACGCATCAGCTCGACGCTCGCCGCGTCCGGACGCATC
GCCCCCTGCGCCACAACGGCCAGTTCACCGACACGCTGCGCCCGCTCGCCTCGCCACG
CGCCACACGCTCGGSSGAGTATCACATAGTGATCCATGAACGCGTTCGCCGCCGCATAG
TCCGCCTGACCCGGATTGCCTCCATAACCCGCCAGCGACGAGAACGTCACGAACAACCTC

CAGCGGCACATCGCGCGTACCCGATCCAGGTTACGACACCCGCCACCTTCGGTGCCAG
CACCGTCCAGTTCGCCACGCTCTTGTT

18) pSPRC107, T3 end.

GAGTTTCGATCCGCTGTTCTTCGGCATCTCGCGCGTGGTGATGGATCCGCAACAGCCTC
CTGATGACTTATGTCTGGAAGGCGCTGGAAGATGCCGGTTATGCGTCGGAGTCGATCTC
GGGCAGCCGCACGCGCTGTTCGTCCGCACCGGGCTGAGCGGTTATCAGAACCTGTTCTC
GGCCGAGGCGCGGGCGGGCGAGGGCTACAGCGCGACTTCGGTGGTGCCGTCCGTCCGGC
CGAACCGGATGAGCTACTTCCTGAACCTGCATGGGCCGAGCGAGCCGATCGAGACGGCC
TGCTCCAGCTCGCTGTGGCGATCAACCGCGGCATCGCAGTGCTGCGCGATGGGACCTGC
GAGATGGCGATCGTCGGCGGCATCAACACCATCGTCACGCCCGACGCGCATGTGAGCTT
CAGCAAGGCGGCATGCTCAGCGAGGACGGCCGCTGCAAGACCTTCTCGGCGGAACGAAC
GGCTACGTGCGCGTGAGGGCGTGCGGATGCTGGTGCTGAAGCGTCTGAGCGACGCGCAG
GCGCCGGCGATGCGATCTATGCCGTGATTGCGGGCGGTTCCGAAAACACGGCGGCCGG
GCGAGTTCGCTGACGGCACCCAACCCGCAGCACAAAGCGGCCCTGCTGATGGACGCGTAT
CGCCGCTCGGGCTCGATCCGCGCACGCTCAGCTATATCGAAGCGCACGGCACGGGTACC
AAGCTCGGCGATCCGGTGGAGATCACCGCCTGAAGAGTGCGTTTGCAAACCTCGGCGTC
GAGTCGGGCGACACGTTCTGCGGGCTCGGTTCCGGTCAAGAGCAACATCGGCCACCTGGA
GCTGGCCGCGG

19) pProt- 123, T7 end.

CCGGTCGCGTGCCCGACGGCCGGCGTGCTGGCGGTGATCGATCGCGAGTGGTTCCGCTT
CGCCATCGAGGCCTTACCACGCCGGCCATCGCGCGATCGCGGACCGAAGCGCTGCCTG
CCGCAGCCGGCCGSGCCACGCTCGCGCCCGCCCGAGGCCCTGGCCGGCGAGAGCGGGG
CCTCAGCGACGCTCGTGATCTCGCCGAGGACACGCCGGAAGTGAGCGATGGCGAGGCG
CGCGCACCGATCGTCGCCGCCATCATGGSCGCATCGAATCGATGCCGGTGGACGAGGCG
CTGTGATCGCGACCGGCGCGTGGAAGTGGCGATCGCCGCGGCGCTCGGCGAGGGCCCTC
ACCGCGCGCCGCTCCGACGCTGGCCGCGCTGACCG

20) pProt- 123, T3 end.

CTGACGTCTTCCTTCAGCGCGGTGGTGCGCTCGGCCACCTGCACCTTGAAGTGGTTGCG
CACGTCTGTCACCCATTGCGTCCAGGCCGGAAGCGGCGTTCTTGAGCTGGATCTGCAC
GCCGTTGCCGACCACCTGCACCTGCGCGGCCGCAAGCCGTGATCGGACAGCGAGCTGGT
CAGCGCGTCGCGCGATGCCGGTGGGCGCCACGCTGCGCGGGCGGCCAGCGAGCGCCTCG
TTGGCCTGCTCGGTCTATCTGCGCGAGTGAGGCGCATGCCGGCAGCGCGGTGGCGATCT
TCTCGCTGTTCTCGGCGGCCGGCGACCACAGCACCGAATACAGCACCACCGCCACCAGC
AGGGCGCCGACGCCAGCAGCAGCTTCTCGCGCGAATTCGTCTGTCAGAACTGGGTG
AGGGTTTCGGTCAGTTCGCGCTTCATGATCCGCTCCGGATGGTCCATTTGCCCGTGCTG
CTGTGATCTCGCCGACAGCCGTTACGCGCCAGG

21) pProt- 125, T7 end.

CGGCTCGGTGCCGAACACGATGCGATGCACTTCGGTGTCGGAGCCGGGATAGTCGATGG
TCGCGCCGGTCACGCCGCCGTCCCAGTTGCGCGGCCGGAACAGGTCGTGCGCGGGCGCC
ACGTCCTCGCCGCGCACGTCTGAAGCGGAACGCGTTGTCGGTGGGCTGCCAGGCGATCGG
CCGCGCGCGCACCTGCSTCGTCGCCGGCGCTCTCGAGCAGCAGCGCCACTCGCTGGGCT
TCCTCGCGCAGGTTCGGTGC GSATTGCGATGCATGGTCAGCGTGGCCGCCGACACGAGGA
TCCCCGCGATCACGAGCACGACGAGCATCTCGAGCAACGTGAAGCCACGCATCCGCCGG
GGcGAAACGCAGCGAGGTTCGTGCCGGTGGTTTCGCATGTCAAACGAAACTGACCAAGAGG
GAAGGGACGGGCGGTGCGATCCGATGCGCCGGGCTTACTGCCAGGAACCGATGTCGGCG
TCGTTGTTCTCGCCGCCTTCCTTGGCGTCGGCGCGTAGCTGAACACGTCGATCTCGCCG
TGCACGCCCGGGTTCAGGTACTTGTACGGGTTGCCCCAGGGATCGTTGGGCAGGCGCTC
GAGGTAGCCGCCGTCTTCCAGTTGTTTCGGCACCGGATCGGTGGTGGGCTTGGCGATCA
GCGATTGCAGGCCCTGCTCCTGGGTTCGGATAGCGGCCGTTGTCGAGGCGGTAGAGCTTG
AGCGCCTGCATGATGGTGCCGATATCCTGCTTGGCGGCCACGCCGTGCCTCGTCCGGGC
GGCTCATGATCTTCGGCACGATCAGCGCGGCCAGGATGCGAGGATCGCGATCACGACCA
TGATTTTCGATCAGCGTGAACCGCGTTGGSACGGCTGGCGGCAGCGCGATTGATCCACGT
TTGCATGAGTGAAACCTCTCTTTCAA AAAACAGTAGTCAACATTGAATGGCGCCGCGGCA
CGTCGCCGCCAGAAGCCGCATTTGTAA

22) pProt- 125, T3 end.

CCGTGGTGCGGCCCAAGAAGGCCTGCACGGCGCGCTGATCTCGCGGATCAAGATCATGG
CGCACGACATCGCCGAGAAACGCCTGCCGCAGATGGCCGGATCACGCTGCGCGCGGCGC
CGGTGGACGTGCGGGTCTCGACGCTGCCACCGGCCACGCGAGCGCGCGGTGCTGCGTC
TGCTGGAAAAGGATGCGCGCCTGAACCTCGAGGCGCTCGGCATGGGCSGACACGCTGG
TGCAGTTTGACAAGCTGATCGGCCGCCCGCACGGCATCGGTGGTCACCGGCCCGACCGC
TCGGAAGACCACCACGCTCTACGCGGCGATGTGCGGCTGGAGACGGCCACCACCAACAT
CATGACGGTCGAGGATCCGATCGAATACGACCTGTCCGGCATCGCCAGACGCAGGTGAA
CGAGCGGATCGGCATGAGCTTCGCGCGCGCTGCGTCGATCCTGCGCCAGGATCCGGACG
TGATCATGATCGGCGAAATCCGCGATCTGGA AACCGCGCAGATCGCCGTGCAGCTCGCT
GACGGGCCACCTGGTGCTGGCCACCCTGCACACCAACGACGCCGCCTCGSCGTACGCG
CCTGACCGACATGGGCGTGGAGCTACCTGCTGGCTTCCTCGCTGCTCGGCGTTGGCGCA
CGGCTGGTGATCCTGGCCCGCATTGCAAGGAACCGCGCGAGGAGGAAGGCCGCGTGAT
CTATCACCCGGTGG

23) pGSP ME H-H 16.7, T7 end.

GACCATCGACCGAGGCGGGCCTCAAAGGGYTTTCAGTGGCGATGGACGATTTCTCGTC
GTCGTGATCGTCGTGCTCGTCGAGGTCCCAGGACGGAAACGGATCGGGCAGCGCGGCC
AGGCTTCCACGCCGGCCGCGTATTTCGGCATCGTCGAGCAGGCAGGCGTCGAATTTGCG
CGCCATTGCSGTCCAGGCCGATGCCGATCAGCACCAGTTCTTGGCGGCGGTTCGCATGCT
GTCGTGTCGGGCGCGCCATGCCAATCGGCGGCGATCTCCTCGAGCAGCGCCGGATCGT
CGTCGGGCCATTCGGCGCGGTCTCTGCcGGCCACACAGAGCCGGCCGGCCGTSASGAC

GCCGCCCCGCTGCGAGASGAGCCGCCGATATCGTTGCGCGTGGCCAGCCAGAAGAAGCC
CTTGCTGCGCACACGCCCTGCCATTCGTTCGTGCAGCAGGGTCCAGAAACGTTGCGGATG
AAACGGTTCGGCGCGCGCGATAGACGAAGTTGCCGATGCCGAACTCGCCGGCCGCGTGCG
TGTGCCCCGAATCCTCGCCGCAATCTTCCCCGTGTTCGTGATCGTGCCGATGCTCGAGC
GAGGCAGCCAGCCCCGGCGCGTTGGCGGGCGCCTCGAAATCGAACAGGCCGGTGTTCGATCA
CCTCGCGCA

24) pGSP ME H-H 16.7, T3 end.

GCTGATGGTGGCGATGCTGGTTCTGCCGGGCCCTACGCTGGCGGCACTCGGTTTCGCCTG
GATCGACCAGCGGCCACCAAGGATGCCGCCGCCACCTCCGATGAGCCGTCCTCGTG
CGGCTCGCGCTACGTCTGCACCCGCCGATTGCACGCATTGAGGCTGCCGGCCGCGACC
GCCGAGCTTCCCTCATCATCCTCATCCGCTTCAGGCGTCCATTCGTGATGCCGAATCTT
CCCGCCTGCTTCCAGGCCGGCTCATCGTCCAGTGAAAGCCCTCCACCAACTGCTCCTGG
CGCTCGTCTTGACGATCCTGATCTACCTCGGCCTGGCGAATTCGCGCTGGACGACTGGT
TCCAGGGCGGCGCGGCTGCTTCGAACGCTGTTTCGATGCCATGGCAAGGCTCGGTATCCA
CGGGGAAGGCGGCATCCTGATCGGCGCCATGCTGGTTCGCCAGCCTTGCGATTGCGGCGA
TGCTGGTTTCGGCTCGCCAGCTTTCGGCGCCGTAATCGCCGGTAACCAGCCACCCCGCGC
CGGTAACACATCGGCTCGCGCCACGTCTTAACCTTACGGCTGATCCGATCGTGTTTCCC
GCGTTCGTTCAGTTACCATTTCGGCCA

25) pGSP ME H-H 2, T7 end.

GGTCAATCGGGGAAAAGCGTCCCAAACACGTCATTCCAGCGCCCGCGAGCTTCCCTCGGG
ACGAAAAAAACCCGCCGCGTCCGTTACAAACGCTGGACTCACCTTGTTCACTGCATCC
TTGAATGCCTTGCCAGCCGTGAACCTGACGGTCTTGGCTGCCGGAATCTTGATGGTTTC
GCCCCTCTTCGGTTGCGGCCGGTACGTGCTGCACGCTTACCCGAGCCGAAGCTGCCGAA
GCCGATCAACTGAACCGCGTCGCCTTTCGACACGGCCTTCTTGATCACCTCGAGGAGCG
TGTCACAGCGTTTCGCCGGTTTGAACCTTGCTGGCGCCCGTCTGTGCGGCGACGGCGTCG
ATCAGTTCCCTGTTTGTTCATTAAAGGTTCCCTTCTCAGGTTAAGTTGACACGAACAGCGC
GGACGCGATTATACGTGCGCAGGCCGTAGCATCGCAGTTTCGCGGCGACGRCCAGGCCAT
CCGGACCGCGCCGNCCTGGTACCCGCGGTGAGCCCTGAGGCGCTTCCGCAGTACGGCGC
TTGCTATGATAGCGAAAACCCAATAACGACAAGGGTTTCAAAGATTTTCATCCGGATT
TTGCCGGATCTTCAGGCCGTTGCCGCATTTTTGCCGCGTGCGACCAGCGTTGTCAAGCG
CCGCAA

26) pGSP ME H-H 2, T3 end.

CCTTGCAAGAACCATCCACCTTGCGCGSTTTCTACCCAGTTGCTCGCCGGCCTGCTCGC
GCGAGACAGGGCCAGTTCATCGGGCATGCAGCGCATCGGCGGCGGCTGCATGCGCATGA
ATTCCGGCTCGTCGCAGGCGAGTTGCAGCAGGCCTTCGCGACTACGGCAGAACGCATGT
CCGGCCGCTTCGAACGCGCGCCATCGCACAGCGTTATTGATAACAGGGCACGCGAGACC

CCGCGCCGAAGTTGTCGTCGATCGCGACCAGCATGGAACACGCCGGCCCTTGCCCCGAGG
CTTCGCTCGCAGTTCGGCATGTGATCGATCAGCGTGAATCGCCAGCCGCGCGCGACGC
TCGACCAGCGCGCAACCTGCCAGGCCCGCGCCGATCACGATCGCGTCGTGCGTATCGGC
GTCGAAGGCGTGCGCTTCGTGTGACGCGAGCTTCCAGCGCGGCCGAAATTCGCCGACCA
GCATCGCGCGCTTGCCCGCAATCCCTCCACCTTGCGATACGAAAAGCCCGCGTTTTCCA
GGGCTCGTTTTACATCGCCGGCGCTCGTGTA

27) pGSP ME H-E 1.7, T7 end.

GCCGACCAGCATCGCGCGCTTGCCCGSAATCCCTCCACCTTGCGATACGAAAAGCCCGC
GTTTTCCAGGGCTCGTTTTACATCGCCGGCGCTCGTGTAGGTGGCGACAKGGCGTGTTT
GTCGGCCAGTTTCGCGAGCGCGCGGAACACCTCGACGGACCAGAGTTTCGGCGTTGCGCG
AGGGCGAAACCATCGAGGTTTAACGCATCGGCACGCAGCACCAGCGAGGGCAGCATCTC
GCGCGCATCGCCGACGCGTCAGTACCACGCGCCCTGCATCGAGTTTCGACGCGATGCAGC
CCCGGCGTGAAATCGGCCAGGCATCGGCGAGTTGGTCGACCAGGCTCGCCTCGATGGTTG
TGCCCGCAACGATATGGCGAGCGGCCTTGCGCAGGTCTTCGCGCGAGAACGGATGTTTC
TCGACCGAGACGAAATGCAGCCGCTCGCTGCGCAAGGATCCGCGCGCCAGGSCCAGGT
CGCGAGGAAGTTGCAGCCGGTGCCGAAGCCGGTCTCGACGATGGTGAAGTGGCGACGCC
GCGCCASTTCGGGCGAGGCGGTTGCGCGCAGGAACACGTGCTCGGCCTGGGCGACGCGCC
GGCGCTATGGTAGATATCGCCGT

28) pGSP ME H-E 1.7, T3 end.

GGTCAAATCGGGGAAAAGCGTGAAACACGTCAATCCAGCGCCCCGCGAGCTTCCTCGGGA
CGAAAAAAAACCCGCCGCGGCCGTTTACAAACGCTGGACTCAGCTTGTTCACTGCATCC
TTGAATTGCCTTGCCAGCCGTGAACCTGACGGTCTTGCTGCTGCCGTTATCTTGATGG
TTTCGCCCCGTCTTCGGTTGCGGCCGGTACGTGCTGCACGCTTACCCGAGCCGAAGCTGC
CGAAGCCGATCAACTGAACCGCGTCGCCTTTCGACACGGCCTTCTTGATCACCTCGAGG
AGCGTGTCCAGCGTTTCGCCGGTTTGAGCCTTGCTGGCGCCCGTCTGTGCGGCGACGGC
GTCGATCAGTTCCTGTTTGTTCATTAAGGTTTCTTCTCAGGTTAAGTTGACACGAACA
GCGCGGACGATTATACGTGCGCAGGCCGTAGCATCGCAGTTCGCGGCGACGACCAGGCA
TCCGGACCGCGCCGSCCTGGTACCCGCGGGTGAGCCCTGAGGCGCTTCCGCAGTACGGC
GCTTGCTATGATA

29) pGSP KS B-B2.2, T7 end.

CGGCGAAATCCGCGATCTGGAAACCGCGCAGATCGCCGTGCAGCTCGCTGAGGGCCACC
TGGTGCTGGCCACCCTGCACACCAACGACGCCGCTCGGCGTCACGCGCTGACCGACA
TGGGCGTGAGCTACCTGCTGGCTTCCCTCGCTGCTCGCGTCTGGCGCAcGGCTGGTGCT
CTGCCGCATTGCAAGGAACCGCGCGAGGAGGAAGGCCGCGTGATCTATACCCGGTGGC
TGCGAGAAGTGCGGCCATTTCGGGCTACACGGGCCGCGCGGTGTCTATGAACTGCTGGT
GATCGACGACGCGATCCGCTCGCTGGTCCACCGCAACGCCCGATTTCGGAAATCCTCGCC
TCGGGCGCTCGAACGGCATGCGCACGCTGCGACGACGCCGAGCGCTGGCTCGCGCAGGG
CAACACCTCGCTAGAGAAGTGCTGCGCGTGACGGGAGGCGCGTAGATGCCGGCATTCCG

TTTCGAAGCGATCGATTCCGGCGGCCGCCCCAGAAGGCGTGATCGAGGCCGACAGCGCG
CGCGCGGCCAGTTGCGCGGCCAGGGTCTCACGCCGCTGGTGGTCGAGCCGGCCGCGAGC
CTCGCGCGATGCSAAAGCAGCSCTGTGATCGGCCGCAAGCTCTCGCAGCGCAGGCCAT
CCTCACGCGCCATCGCCAGCCTCTTGACCGCCGGGCTGCCGCTCGGAAATCGCTGGCGG
TGCTCACCGAG

30) pGSP KS B-B2.2, T3 end.

GCAGCCCGGGGATCTATCCCTCGCCCTCTTCGCGGCCTTGTGCGACGGCCACCTACTG
GGCCATCACGTCTCGGGCATCAGGCCCCGCTACCCGCCGCCGCCGCGCTCCCTTGCG
CACGGAGGACGCTGCCGCGCTGTTGCGCGGGCAGTCACGCACAACCCCGTGAGGACAT
CCACCTGTTTCGGCATCCTCGCGCTGCCAGGSGCCGCGATCGTCGGCATCGGCGGATCC
GGCCCGCACCATCACGCTGGGGCGACATCTCCAGGGCACCAAGCTCGCCGAGGTCCGC
GCCCGCTCGATCGTGATCGACCGCAACGGCGCGCATGCCGAGATCTTCCTGCCGGCCAA
CACGCCCGGGCCCGCCATCTACGTGCGCTGAGCCGTTCCCTTCCATAACGCTCATCGCC
GACGTTTCATTCCGGCAACGGTCGCGATCGGCTTTGCCTTGATCCCTTACTGCACCATG
TTGTTTCAGCTCGATGATCGGCAGCATCACGGCCAGCACGATCACCAGCACGATGCCGCC
CATCGCCAGGATCAGGAGCGGCTCGAGCAGGCTGGTCATGAACATGGTGCGGCGCTCGA
GTTTCGCGCGCCTCACCTCGGCGGCGGGTCGAGCATGGTGGTGACGTCGCCGGTGGCTCG
CCGAGCGGATCAGGTGCACCAG

31) pGSP KS B-B7.2, T7 end.

TCGACCGTCATGATGTTGGTGGTGGCCGTCTCCAGCCGCGACATCGCCGCGTAGAGTGG
TGGTCTTGCCCGAGCCGGTCGGCGGTGACCAGCACGATGCCGTCGGCGGCCGATCAGCT
TGTCAAACTGCACCAGCGTGTCGCGCCCATGCAGCGCCTCGAGGTTTAGGCGCTGCGCA
TCCTTTTCCAGCAGACGCAGCACCGCGCGCTCGCCGTGGCCGGTGGGCAGCGTCGAGAC
CCGCACGTCCACCGGGCGCCGCACGCGCAATCCGGCNATCCTGCGGCAGGCGTTTCTCG
GCGATGTCGAGCTGCGCCATGATCTTGATCCGCGAGATCAGCGCGCCGTGCAGGGCCTT
CTTGGGCGCACACGTGCGCATGCCGTCGACGCGAAAGCACACCGAGGCGTTCTCGA
AGGGCTCGATGTGGATGTGGAAGCCTGCTCGCGCGCGGCTGGGTGAGCAGGGCGTTG
ATCATGCGGATGATCGGCGCGTCTGCTCCGATTCCAGCAGGTCCTCGACTTCCGGGAT
GTCTTGATCAGCCGCGACAGGTGACCTCGCCTTCCACCTCGCCGACCACCTGCGCCG
CGCTGCCGTCCTGGCGCGCATAGGGTGTTGATGGCCTGCGCGACGTCGTCGGCCGGCAG
CGAGCNTCAACGCGCGAAGTTGCGTGCCACCTCGGCCAGCGCG

32) pGSP KS B-B7.2, T3 end.

CATCGACCGAGGCGGGCCTSAAGGGTTTCAGTGCGGATGGACGATTTCCCTCGTCGTCGT
GATCGTCGTGCTCGTCGAGGTCCCAGGACGGAACGGATCGGGCAGCGCGGCCAGGCT
TCCACGCCCGGCCGCGTATTCGGCATCGTCGAGCAGGCAGGCGTCGAATTCGCGCGCCA
TTGCGTCCAGGCCGATGCCGATCAGCACCAGTTCTTGGCGGCGGTGCGATGCTGTCGTC
GTCGGGCGCGCCATGCCAATCGGCGGCGATCTCCTCGAGCAGCGCCGGATCGTCGTCGG

GCCATTTCGGCGCGGTCTCTGCGGCCCCACCAGAGCCCCGGCCGGCCCGTGAGACGCCGCCCG
CCTGCGAGAGAGCCGCCGATATCGTTGCGCGTGGCCAGCCAGAAGAAGCCCTTGCTGCG
CAACGCCCTGCCATTTCGTCTGTCAGCAGGGTCCAGAAACGTTGCGGATGAAACGGTCGG
CGCGCGCGATAGACGAATTGCCGATGCCGAACTCGCCGGCCGCGTGCGTGTGCCCGCAA
TCCTCGCCGCAATCTTCCCCGTGTTTCGTGATCGTGCCGATGCTCGAGCGAGGCGAGCCA
GCCCCGGCGCGTTGGCGCTCGAAATCGAACAGGCCCGGTGTCGATCACCTCGCGCAGCACC
GCGCCGAAGCGC
